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Microdialysis sampling of the isothiazolone, PD-161374, and its thiol and disulfide metabolites

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Abstract

A method based on microdialysis sampling combined with high-performance liquid chromatography (HPLC) has been developed for monitoring the anti-HIV agent PD-161374 (isothiazolone) and its thiol and disulfide metabolites in blood. It was demonstrated that unlike blood withdraw and extraction, microdialysis sampling can preserve the distribution among the isothiazolone and its metabolites in blood. The use of a narrow-bore HPLC system, combined with the relatively high probe extraction efficiency ($\sim 50\%$) from the flexible probe design in this work, allows the direct and quantitative determination of the drug and its major metabolites at submicromolar level. © 2000 Published by Elsevier Science B.V.

Keywords: Microdialysis sampling; Isothiazolone; PD-161374

1. Introduction

Among the compounds selected by the National Cancer Institute HIV Screening Program are several isothiazolones and disulfide benzamides [1–4]. These compounds have been shown to possess anti-HIV activity at low micromolar concentrations. 3-Methyl-2-(3-oxo-3H-benzo-[d]-isothiazol-2-yl)-pentanoic acid (PD-161374) is an isothiazolone that is representative of this class of compounds. An important characteristic of these compounds is that the isothiazolone form exists in equilibrium with the thiol and disulfide forms as shown in Fig. 1 [3,4].

Conventional methods of sampling these compounds for pharmacokinetics studies employ blood withdrawal. Analysis requires protein precipitation and solid-phase extraction. This can severely disturb the distribution among the isothiazolone, thiol, and disulfide forms of the compound. The current method therefore converts the compound completely to its thiol form for analysis. Critical information about the distribution of the compound between its various forms in vivo is therefore lost using this method.

Microdialysis sampling has found a wide range of applications in biological and pharmacological

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studies [5,6]. In microdialysis sampling a probe consisting of a short length of small diameter hollow fiber membrane is implanted at the desired sampling site. The fiber is slowly perfused with a sampling solution (perfusate) that closely matches the matrix at the sampling site. The dialysis membrane has a molecular weight cutoff (typically around 5000–20 000) that allows only small, soluble molecules in the sample to diffuse across the membrane into the fiber lumen, where they are swept away by the perfusate and collected as dialysate for analysis. Macromolecules, such as proteins, are excluded from the dialysis probe. Therefore microdialysis samples can be directly injected into an HPLC system without need for protein precipitation or other sample handling. Microdialysis sampling should be capable of sampling PD-161374 without perturbing the distribution between the various forms of the compound. This is in contrast to the conventional method of blood withdrawal and extraction prior to analysis, where the extraction step significantly alters the distribution of the form of the compound. The goal of this work was to develop a method based on microdialysis sampling and HPLC analysis for studying the pharmacokinetics of PD-161374 and its thiol and disulfide forms without disturbing the in vivo distribution.

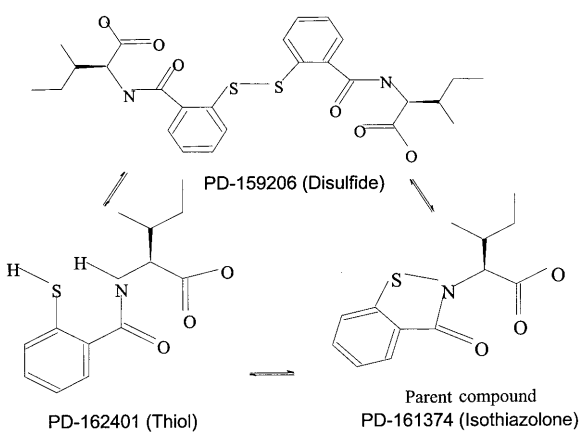

Fig. 1. Equilibrium between PD-161374 (isothiazolone), PD-162401 (thiol), and PD-159206 (disulfide).

2. Experimental

2.1. Chemicals and reagents

HPLC grade acetonitrile was obtained from Fisher Scientific (Fair Lawn, NJ). Ionated trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL). Certified acetic acid was obtained from Mallinckrodt Chemical (St. Louis, MO). 3-methyl-2-(3-oxo-3H-benzo-[d]-isothiazol-2-yl)-pentanoic acid (PD-161374, isothiazolone), 2-{2-[2-(1-carboxy-2-methyl-butylcarbamoyl)-phenyldisulfanyl]-benzoylamino}-3-methyl-pentanoic acid (PD-159206, disulfide), 2-(2-mercapto-benzoylamino)-3-methyl-pentanoic acid (PD-162401, thiol) were provided by Parke–Davis Pharmaceutical Research. All other chemicals were reagent grade or better and were used as received. Deionized water (Barnstead, Dubuque, IA) was used for the preparation of all the solutions.

Ringer's solution consisted of 147 mM NaCl, 4 mM of KCl and 2.5 mM of $CaCl_2$. Stock solutions (1 mg/ml) of the isothiazolone, thiol and disulfide were prepared daily. For the isothiazone, 1 mg was weighed and dissolved in 1 ml of the Ringer's solution. For the thiol, 1 mg was weighed and dissolved in 0.3 ml of 6 N acetic acid and diluted to 1 ml with Ringer's solution. For the disulfide, 1 mg was weighed and dissolved in 0.3 ml of 0.1 N NaOH and diluted to 1 ml with Ringer's solution. Standard solutions were obtained by dilution of the stock solution to the desired concentrations with Ringer's solution immediately prior to use. The pH for all standard solutions was adjusted to 7.2.

2.2. Liquid chromatography

The liquid chromatographic system consisted of two Shimadzu LC-6A pumps, an SCL-6B system controller and an SPD-10A UV-visible absorbance detector (Shimadzu Scientific Instruments, Inc., Columbia, MD) with an 8-μl standard cell. Separations were performed at ambient temperature with a Zorbax Rx-C8 5 μm particle size, 2.1 × 150 mm column (MAC-MOD Analytical, Inc., Chadds Ford, PA). A 2.1 × 12.5-mm 300SB-C8 5 μm particle size guard column

(MAC-MOD Analytical, Inc.) was placed between the injector and the analytical column. The mobile phase flow rate was 0.2 ml/min. Samples were injected with a Rheodyne 7125 valve (Alltech Associates, Deerfield, IL) with a fixed volume 10 μl PEEK sample loop. A detection wavelength of 250 nm was used for all experiments. Chromatograms were acquired and analyzed with Perkin–Elmer Turbochrom software.

The separation was achieved by gradient elution. Mobile phase A was 31:69 (v/v) acetonitrile/0.1% TFA. Mobile phase B was 70:30 (v/v) acetonitrile/0.1% TFA. Gradient elution was performed by running 100% mobile phase A for the first 14 min, ramping from 100% mobile phase A to 100% mobile phase B over 1 min (from 14 to 15 min) and maintaining 100% mobile phase B for the next 5 min. The column was re-equilibrated with mobile phase A for 5 min between runs.

2.3. Microdialysis sampling

The microdialysis sampling system consisted of a CMA/100 syringe pump, a CMA/140 microfraction collector (Bioanalytical Systems, Inc/Carnegie Medicin, West Lafayette, IN), a 1000-μl Hamilton syringe and a microdialysis probe. The flexible probe design employed in this work was fabricated using the procedures described previously [7]. It is composed of a 1-cm length of regenerated cellulose membrane, 200 μm i.d. and 222 μm o.d. (CGH Medical, CO) and two pieces of 75 μm i.d., 150 μm o.d. fused silica capillaries (Polymicro Technologies Inc., Pheonix, AZ) tubing. The inlet of the probe was connected to the perfusate syringe using PE-50 tubing. A BAS Beekeeper awake animal system (Bioanalytical Systems, West Lafayette, IN) was used to house the experimental animals during pharmacokinetics experiments.

2.4. Microdialysis probe calibration

Microdialysis probe extraction efficiencies were determined both in vitro and in vivo. The extraction efficiency was determined in vitro by a recovery experiment. For the recovery experiment, the probe was placed in a stirred standard solution of known concentration (5–50 μg/ml) thermostated at 37°C and perfused with Ringer's solution at a flow rate of 1 μl/min. The extraction efficiency by recovery (EE_R) was calculated as:

$$EE_R = 100^* C_d / C_s$$

where C_d is the concentration in the dialysate and C_s is the concentration in the standard solution.

The extraction efficiency of each probe was also determined in vivo by a delivery experiment. For the delivery experiment, a 25-μg/ml standard solution of the test compound in Ringer's solution was perfused through the probe at 1 μl/min. The extraction efficiency by delivery (EE_D) was determined from the change in concentration in the dialysate as:

$$EE_D = 100^* (C_p - C_d) / C_p$$

where C_p is the concentration in the perfusate and C_d is the concentration in the dialysate.

2.5. Surgical procedures

Male Sprague–Dawley rats weighing between 350 and 450 g were used for all experiments. Rats were supplied by the University of Kansas Animal Care Unit breeding colony. All surgical procedures were performed according to Animal Use Statements approved by the University of Kansas Institutional Animal Care and Use Committee. Before the surgery, the rats were initially anesthetized by inhalation of the anesthetic isoflurane (Aerrane, Fort Dodge Animal Health, IA). A mixture of 95 mg/kg Ketamine (Ketaset, Fort Dodge Animal Health, IA)/5 mg/kg Xylaxine (Rompun, Bayer Corporation, KS) was then administered subcutaneously. This combination provides about 45 min of surgical anesthesia, and the rats were fully recovered in 5 h. The back of the neck and the right of the rat's shoulder were shaved and sterilized. Small incisions through the skin were made at the above two areas. The jugular vein was exposed on a flat scoopula and a triangular nick was made into the vein. The microdialysis probe and cannula tubing (0.28×0.61 mm polyethylene tubing) filled with Ringer's solution containing (1000/1 v/v) heparin (Elkins–Sinn, Inc. NJ) were inserted through the nick

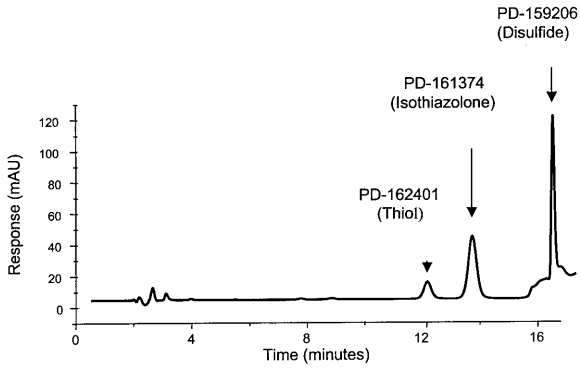

Fig. 2. Chromatogram of PD-161374 (isothiazolone), PD-162401 (thiol), and PD-159206 (disulfide). Column: Zorbax Rx-C8, 2.1 × 150 mm, 5 μm particle size; Mobile phase A: 31:69 acetonitrile:0.1% trifluoroacetic acid (v/v); Mobile phase B: 70:30 acetonitrile:0.1% trifluoroacetic acid (v/v); Gradient: 100% A for 0–14 min; 100% A to 100% B over 14–15 min; 100% B for 5 min. Flow rate: 0.2 ml/min; detection: UV at 250 nm; injection volume: 10 μl.

Table 1
Dependence of percent extraction efficiency in vitro on concentration

Conc (μg/ml)	Isothiazolone	Thiol	Disulfide
5	–	45.5 ± 0.6	–
10	64.0 ± 2.3	47.2 ± 1.7	32.9 ± 0.7
25	61.5 ± 0.6	46.2 ± 0.3	35.6 ± 1.2
50	62.7 ± 1.1	ppt[a]	33.8 ± 0.9

[a] Precipitated in 50 μg/ml standard solution.

about 2 cm. The cannula tubing was inserted about 5 mm further than microdialysis tubing. The probe and cannula tubing were ligated to the jugular vein. The tubing was then threaded under the skin and out the incision on the back of the neck. The incisions were closed with surgical staples. The rat was placed into the BAS Beekeeper system house and the inlet and outlet of probe were connected to the perfusion pump and fraction collector through a counter-balanced arm. In vivo experiments were performed by perfusing Ringer's solution through the probe at a rate of 1 μl/min and continuously collecting the dialysate over 10-min intervals. Blank dialysates were collected and checked with HPLC to make sure there were no chromatographic interferences before the drug was administered. A 0.3–0.4-ml dose of 12 mg/ml PD-161374 in Ringer's solution was administered through the jugular vein cannula. The dose volume was adjusted based on the animal's body weight to give a final dose of 10 mg/kg body weight. After dosing the jugular vein cannula was rinsed with 0.2 ml of Ringer's solution containing 0.1% (v/v) heparin.

3. Results and discussion

3.1. Liquid chromatography

Fig. 2 shows a typical chromatogram of PD-161374 (isothiazolone), PD-162401 (thiol), and PD-159206 (disulfide). Gradient elution was used so that the isothiazolone and the thiol were separated without having the disulfide retained too long. Mobile phases containing more than 31% acetonitrile could not resolve the isothiazolone and thiol. However, with isocratic elution with such a weak mobile phase the disulfide was retained in excess of an hour. The use of gradient elution resulted in retention times for PD-161374 (isothiazolone), PD-162401 (thiol), and PD-159206 (disulfide) of 12.5, 14.0 and 17.0 min, respectively.

The linearity of the method was evaluated over the concentration range of 0.02–20 μg/ml, 0.03–10 μg/ml and 1.6–25 μg/ml for PD-161374 (isothiazolone), PD-162401 (thiol), and PD-159206 (disulfide), respectively. The response was linear for all three compounds over the concentration range tested. A detection limit of 6.0 ng/ml (based on S/N of 3) for PD-161374 (isothiazolone) and PD-162401 (thiol) and 20.0 ng/ml for PD-159206 (disulfide) was achieved.

3.2. Microdialysis probe calibration

The microdialysis probes were characterized in vitro prior to implantation and use in pharmacokinetics experiments. The concentration dependence of the EE_R was first investigated. The EE_R in vitro was independent of concentration over the range of 5–50 μg/ml (Table 1). The stability of probe behavior was investigated by comparing

the EE in vitro before implantation and after being removed from the jugular vein after the pharmacokinetics experiment. The EE_R of the probes used in these studies were not significantly different after removal from the jugular vein relative to before implantation (Table 2). This is an indication that there was no adhesion to the probe surface changing the dialysis characteristics. For the same microdialysis probe, the isothiazolone (PD-161374) exhibited a higher EE than the thiol (PD-162401) and the thiol had a higher EE than the disulfide (PD-159206).

The extraction efficiency was also determined in vivo by a delivery experiment. The EE in vivo was in good agreement with the EE in vitro for the isothiazolone (PD-161374) and thiol (PD-162401) as seen in Table 2. This is the behavior expected for a microdialysis probe implanted in the jugular vein [8,9]. The significantly higher EE in vivo for the disulfide (PD-159206) compared to the EE in vitro was not expected. It is likely caused by the very high protein binding of the disulfide. High protein binding results in a low concentration of free disulfide outside the membrane and a correspondingly steeper concentration gradient across the membrane. The steep concentration gradient results in a higher EE. Based on these observations, probes must be calibrated in vivo for quantitative determination of the disulfide.

Table 2
Effect of use on percent extraction efficiency

	Thiol	Isothiazolone	Disulfide
Probe # 1			
Before use	47.3 ± 1.8	59.6 ± 1.8	34.8 ± 0.8
After use	51.7 ± 4.4	62.3 ± 0.9	35.2 ± 1.4
In vivo	50.2 ± 0.1	61.6 ± 0.2	89.3 ± 0.1
Probe # 2			
Before use	44.0 ± 1.9	65.0 ± 0.9	39.3 ± 0.5
After use	42.1 ± 1.1	62.2 ± 2.8	36.6 ± 0.9
In vivo	46.1 ± 1.5	65.0 ± 1.2	84.8 ± 0.3
Probe # 3			
Before use	46.8 ± 1.1	60.4 ± 0.1	31.9 ± 1.1
After use	47.2 ± 0.7	60.1 ± 0.5	34.2 ± 1.8
In vivo	45.2 ± 0.9	60.9 ± 1.1	81.9 ± 1.5

3.3. Impact of microdialysis sampling on the equilibrium

The primary aim of this work was to develop a sampling technique that does not alter the relative concentrations of the isothiazolone, thiol, and disulfide forms of the compound. The sample preparation steps to precipitate protein and extract the analytes are typically responsible for altering the equilibrium of this system. Because microdialysis sampling does not require these steps, it should be capable of monitoring the concentrations of the three compounds without changing their distribution. This was tested in vitro in a system in which microdialysis sampling and direct removal of aliquots could be done simultaneously. A microdialysis probe was placed into a stirred solution containing a standard solution. Dialysis samples were collected over 60-min intervals and immediately injected into the HPLC system for analysis. At the mid-point of collection of each dialysis sample an aliquot was directly removed from the sample and injected into the HPLC system for analysis. The concentrations of all three compounds (isothiazolone, thiol, and disulfide) were determined in both the dialysis samples and the directly sampled aliquot.

In one experiment, an initial mixture of 10 μg/ml PD-162401 (thiol), and 10 μg/ml PD-159206 (disulfide) in Ringer's solution was used as a model system. As expected, the isothiazolone rapidly formed when the thiol and disulfide were mixed [3,4]. The change in concentration of each component as a function of time from both the dialysis samples and the directly sampled aliquots is shown in Fig. 3. The change in concentration of all of the compounds in the dialysis samples tracks the change in concentration observed in the directly sampled aliquots. In addition, each pair of dialysis sample and directly sampled aliquot represents a recovery experiment. If the relative concentrations of the isothiazolone, thiol, and disulfide were changed by the microdialysis process this would result in an apparent change in EE. As seen in Fig. 3, the EE for all three compounds is constant throughout the experiment even as the concentrations of the three compounds change dramatically.

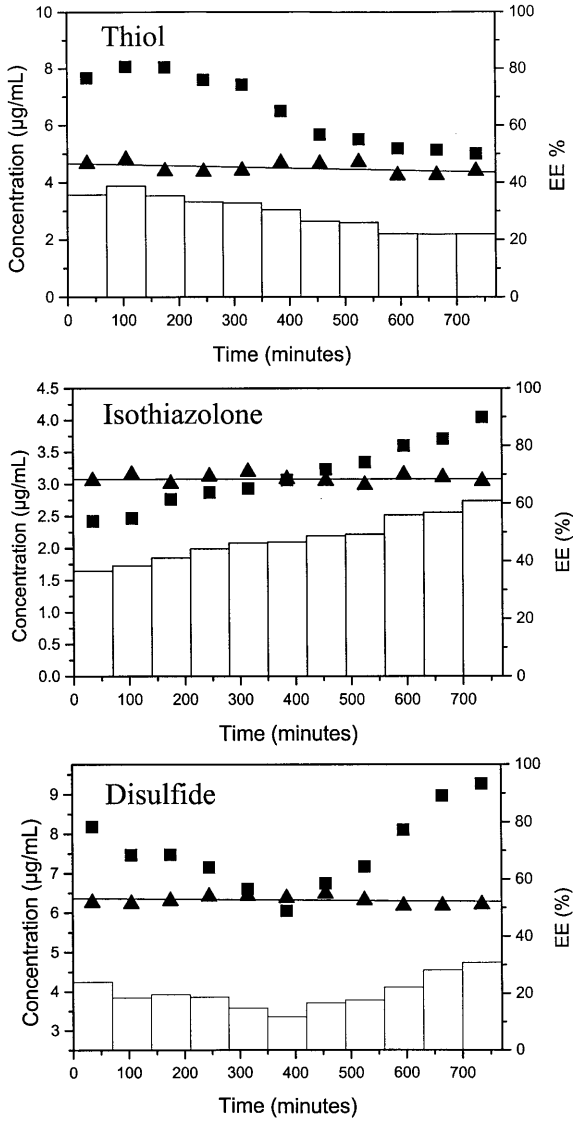

Fig. 3. Concentration and percent extraction efficiency (EE%) versus time for an initial mixture of 10 μg/ml PD-162401 (thiol) and 10 μg/ml PD-159206 (disulfide). Symbols: bars are the dialysis samples; ■ are directly sampled aliquots; ▲ are the extraction efficiencies.

In a second experiment, an initial mixture of 10 μg/ml PD-162401 (thiol) and 4 μg/ml PD-161374 (isothiazolone) was used. In this case, disulfide was formed immediately upon mixing of the thiol and isothiazone. As with the first mixture, changes in the concentration of all three forms of the compound in the dialysis track those in the directly sampled aliquots (Fig. 4). Also as before, the EE determined as the ratio of the concentration in the dialysis sample to the concentration in the directly sampled aliquot did not change over time or as the concentration of the individual compounds changed. These results indicate that

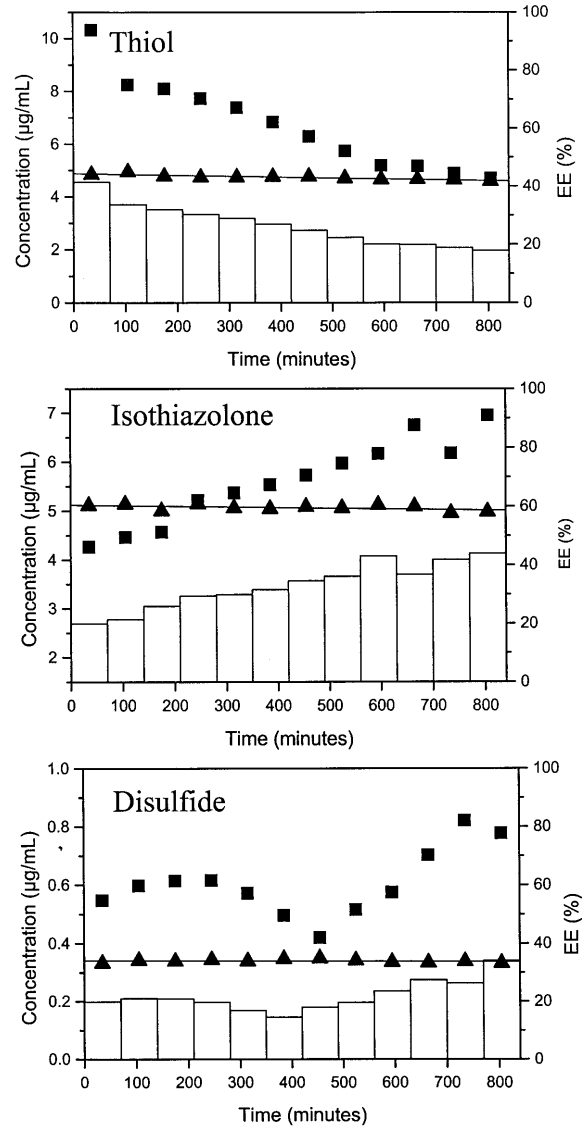

Fig. 4. Concentrations and percent extraction efficiency (EE%) versus time for an initial mixture of 10 μg/ml PD-162401 (thiol) and 4 μg/ml PD-161374 (isothiazolone). Symbols: as in Fig. 3.

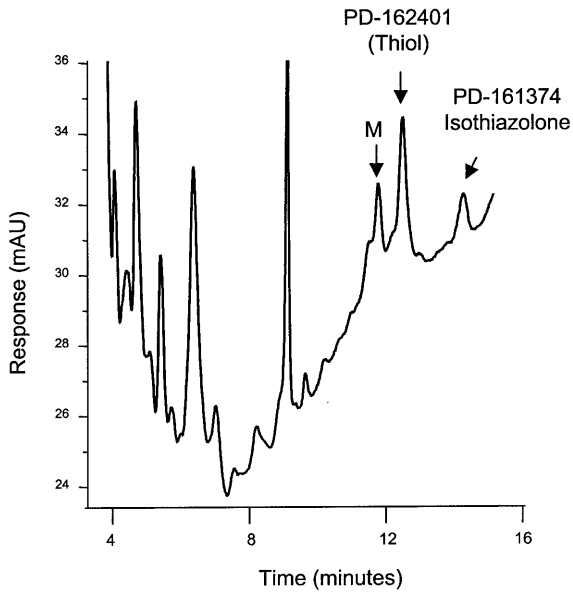

Fig. 5. Typical chromatogram of a blood microdialysis sample after dosing PD-161374. Chromatographic conditions as in Fig. 2.

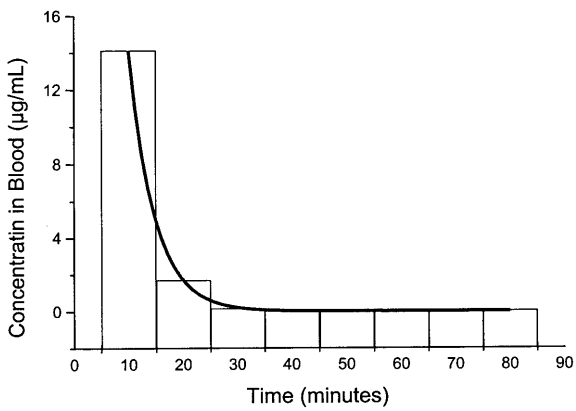

Fig. 6. Concentration-time curve of PD-161374 (isothiazolone) in the blood microdialysis samples.

microdialysis sampling can be used to monitor the concentrations of all three forms (the isothiazolone, thiol, and disulfide) without changing their relative distribution.

3.4. Pharmacokinetics studies

The final step was to use microdialysis sampling to study the pharmacokinetics of all three compounds in blood following a dose of PD-161374 (isothiazolone). A microdialysis probe and dosing cannula were implanted into the jugular vein of a rat. After the animal had recovered from surgery, a 10-mg/kg dose of PD-161374 in Ringer's solution was administered through the dosing cannula as a bolus. Dialysis samples were collected over 10-min intervals and immediately injected into the HPLC for analysis. Fig. 5 shows a representative chromatogram of a dialysis sample collected from 30 to 40 min following dosing. The thiol was the main form of the compound with a small amount of the isothiazolone detected in the blood. The disulfide was not detected in any of the microdialysis samples. An unknown peak (M) that eluted immediately prior to the thiol was also detected. This peak is likely a mixed disulfide metabolite of PD-161374 with an endogenous thiol such as glutathione. Concentration-time curves for the isothiazolone and thiol are shown in Figs. 6 and 7, respectively.

The pharmacokinetics of the parent compound, PD-161374 (isothiazolone) was evaluated using a one-compartment, first-order open model with intravenous dosing [10,11]. A model of metabolite formation and elimination was employed for the thiol. The pharmacokinetics parameters calculated are summarized in Table 3.

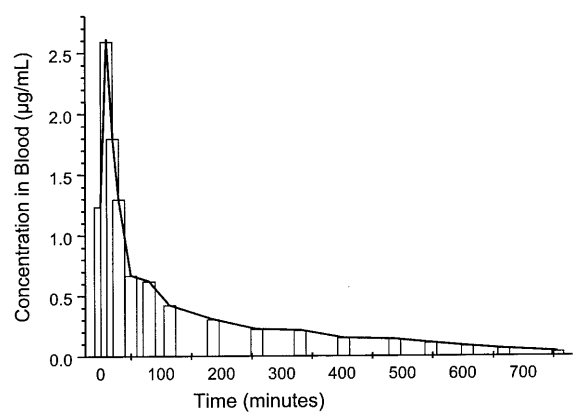

Fig. 7. Concentration-time curve of PD-162401 (thiol) in the blood microdialysis sample.

Table 3
Pharmacokinetic parameters derived from microdialysis sampling data[a]

	Awake rat #1	Awake rat #2	Awake rat #3	Average	Stdev
Isothiazolone					
$t_{1/2}$	6.70	9.29	8.70	8.23	1.36
k_{el}	6.21	4.48	4.78	5.16	0.92
AUC	98.96	120.28	109.98	109.74	10.66
Cl_p	38.80	33.35	33.64	35.26	3.07
Thiol					
$t_{1/2}$	149	139	167	151.67	14
k_{me}	0.28	0.30	0.25	0.28	0.03
k_m	0.47	0.25	0.43	0.38	0.12
k_e	5.74	4.22	4.35	4.77	0.84
AUC	228.46	227.66	303.46	253.19	43.53

[a] $t_{1/2}$ is the half-life (min); k_{el} is the elimination rate constant (h^{-1}); Cl_p is the plasma clearance (ml/min); k_{me} is the first-order rate constant of excretion of metabolite (h^{-1}); k_m is the first-order rate constant for formation of metabolite (h^{-1}); k_e is the first-order rate constant of excretion of drug (h^{-1}); and AUC is the area under concentration-time curve (μg/min per ml).

4. Conclusions

Microdialysis sampling was capable of monitoring the concentrations of three compounds involved in a complex equilibrium without changing their relative concentrations. This allowed the determination of the pharmacokinetics of each compound individually rather than of total compound as necessitated by previous sampling methods.

Acknowledgements

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