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On-line determination of fluconazole in blood and dermal rat microdialysates by microbore high-performance liquid chromatography

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Abstract

To study the distribution of fluconazole in the dermis of the rat, on-line microdialysis using double-site sampling coupled with a microbore HPLC system was developed. The chromatographic conditions consisted of a mobile phase of 20 m*M* diammonium phosphate–acetonitrile (75:25, v/v, pH 7.0) pumped through a microbore C_{18} column at 40 μl/min. The eluent was monitored with UV detector with UZ flow cell (30 mm path length) at 210 nm. A microbore 10-port pneumatic valve fitted with two loops of 1 μl was used to collect and directly inject microdialysates from jugular and dermal probes. The retention time was 5.8 min for fluconazole and 10.1 min for its fluorinated analog, UK-54373 used as a retrodialysis marker. The assay was precise, with inter- and intra-assay relative standard deviation values of 0.64 and 0.71%, respectively, and with a good linearity ($r=0.999$) in the range of 0.15–20 μg/ml with only 1 μl injected onto the column. The LOD and LOQ values for fluconazole were 0.100 and 0.150 μg/ml, respectively. The applicability of the method was demonstrated by studying the disposition of fluconazole in blood and dermis following i.v. bolus at a dose of 10 mg/kg.

Keywords: Microdialysis; Fluconazole

1. Introduction

Fluconazole, [2-(2,4,-difluorophenyl)-1,3-bis(1H-1, 2,4,-triazol-1-yl)propan-2-ol], is an orally active antifungal agent, which is used in the treatment of superficial and systemic candidiasis and in the treatment of cryptococcal infections in patients with the acquired immunodeficiency syndrome (AIDS). It acts by blocking the synthesis of ergosterol, an essential component of the fungal cell membrane [1]. Like antibiotics, the determination of unbound active concentrations of antifungal agents at the site of infection, particularly in the dermis, is more relevant that the measurement of plasma concentrations. Techniques that measure tissue drug concentrations directly are more appropriate for pharmacokinetic

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studies than indirect estimations of tissue levels from plasma concentrations. A limited number of techniques are available for direct assessment of drug concentrations in dermis [2]. These include the skin blister fluid method, stripping method, and biopsy followed by tissue homogenization. However, ethical considerations, cost and lack of reproducibility limit the applicability of these techniques in pharmacokinetics. Moreover, these techniques only allow determination of the total concentrations of drug, whereas the pharmacological activity relates to the unbound concentration.

Overcoming the inherent limitations of the abovementioned techniques, microdialysis is an in vivo sampling technique that has become very popular in conventional pharmacokinetic studies [3]. The principle of the technique is based on the passive diffusion of compounds down a concentration gradient across the semipermeable membrane of a dialysis fiber. Microdialysis has several advantages for in vivo sampling of drugs. No fluid is removed, so continuous sampling can be performed with small laboratory animals [4,5] without affecting the pharmacokinetic profiles while allowing determination of concentration–time profiles with high temporal resolution. Samples are relatively clean and protein-free because the membrane is only permeable to small molecules. By excluding macromolecules, microdialysis permits sampling of the analytes in their nonprotein bound form thus simplifying any sample clean-up procedures. Direct analysis of microdialysates by on-line injection into the HPLC system allows automating the pharmacokinetic studies in order to reduce the stress of the animal and the variability of the pharmacokinetic parameters. Moreover on-line analysis eliminates the problems associated with evaporation and sample transfer. For the application of microdialysis in pharmacokinetic studies, the in vivo determination of the relative recovery allows the conversion of microdialysate concentrations into extracellular concentrations [6]. Several approaches have been proposed to determine the in vivo recovery of the analyte by the microdialysis probe: e.g. the stop flow method [7], the extrapolation to zero flow method [8], the point of no net flux [9] and the retrodialysis method [10]. Presently, the retrodialysis method is more often used because unlike the others, it is faster and more convenient. The most important advantage of this technique is that fluctuations in the recovery of the probe during the experiment are taken into account by continuous retrodialysis of the marker during the entire experiment. Besides this, microdialysis presents an important analytical challenge. Low perfusate flow-rates (0.5–5 μl/min) are used to enhance the relative recovery of analytes by the probe. This results in small sample volumes generally containing small absolute amounts of analyte. This is particularly true for drugs with high protein binding. The moderate lipophilicity ($\log P_{\text{octanol}} = 0.5$) [11] and the low protein binding of fluconazole constitute important advantages for studying its distribution in dermis using microdialysis sampling.

The currently available assays for fluconazole are bioassays [12,13], gas chromatography (GC) assays using electron-capture, nitrogen-selective or ion trap detector [14] and high-performance liquid chromatographic (HPLC) methods. The reported methods have some disadvantages. Bioassay is a time-consuming technique and is not very reliable since medium conditions are known to influence the in vitro activity of fluconazole [15]. The GC methods are very sensitive, but the sample preparation is sometimes laborious [14] and the validated concentration range is too narrow (0.01–1.5 μg/ml) for use in pharmacokinetic studies [16]. The most commonly used HPLC method [17] includes an extraction step using ethyl acetate, followed by chromatography on reversed-phase columns, elution with an isocratic mixture of methanol and phosphate buffer and a detection at 260 nm. Hosotsubo et al. [18] used a simple deproteinisation of biological samples, an acetonitrile–water mobile phase and detection at 210 nm. Recently, the HPLC fluconazole assay was improved using a mixed phase column [19] or a solid-phase extraction [20] as the sample preparation method.

In the HPLC assay described by Flores-Murrieta [21] a conventional column (3.9 mm, I.D.) was used to determine fluconazole. Because fluconazole is a poorly absorbing compound, the limit of quantification of 0.1 μg/ml was obtained by injecting a large sample volume (80-μl) incompatible with the microdialysis technique. Indeed, the temporal resolution of a microdialysis study depends on the combination of the perfusate flow-rate through the microdialysis

probe and the sample volume requirements of the analytical technique [6] and the time of analysis. By using a narrow-bore column (2.1 mm, I.D.) the injected volume of the sample was reduced to 10 μl by Elmquist [22] to obtain a sufficient sensitivity for pharmacokinetic studies with microdialysis. The microdialysates were injected every 20 min in order to obtain a sample volume of 10 μl collected from a probe which was perfused at a flow-rate of 0.5 μl/min.

In our laboratory we have initiated a study focusing on the distribution of fluconazole in cutaneous tissues in the awake, freely moving rat by using microdialysis. In order to analyze on-line small volumes of microdialysate with a sufficient sensitivity, a new HPLC method for fluconazole using a microbore column had to be developed. The principal attribute of microbore column (1 mm, I.D.) is the high mass sensitivity required to work with very small biological samples that contain limited quantities of the solute to be analyzed [23–25]. The present paper describes the first microbore HPLC method with ultraviolet detection for on-line determination of fluconazole in small volume of microdialysates collected alternatively from two probes implanted in the jugular vein and dermis in awake rats.

2. Experimental

2.1. Reagents

Fluconazole and UK-54373, a fluorinated analog of fluconazole used as retrodialysis marker (Fig. 1), were kindly provided by Pfizer (Sandwich, UK). Acetonitrile HPLC ultra gradient grade and methanol HPLC grade were purchased from J.T. Baker (Deventer, The Netherlands). Water was ultrapure (Sation 9000, Vel, Leuven, Belgium). Sodium chloride, disodium hydrogen phosphate dihydrate and diammonium hydrogen phosphate of analytical grade were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation

The high-performance liquid chromatography (HPLC) system consisted of a Kontron Instruments

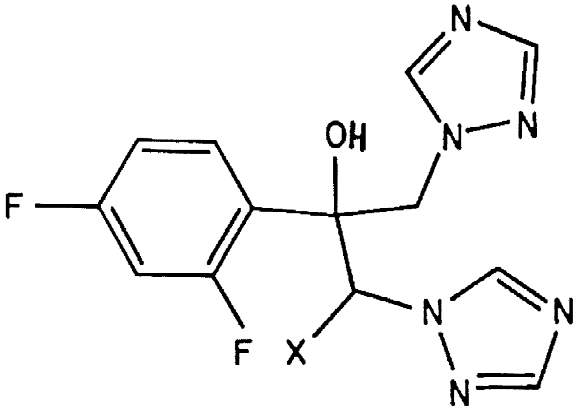

Fig. 1. Chemical structures of fluconazole and the retrodialysis marker, UK-54373. X is H in fluconazole and F in UK-54373.

Model 422 pump (Kontron, Milan, Italy), an Ultimate™ UV–vis detector (LC Packings, Amsterdam, The Netherlands) equipped with an UZ-View Capillary flow cell (LC Packings), with a path length of 30 mm, an illuminated volume of 540 nl, a data acquisition system Kromasystem 2000 Version 1.83 (Bio-Tek Kontron Instruments, Milan, Italy) and a microbore 10-port valve Cheminert™ C2-1000D with 0.2 mm diameter channels (Valco Instruments, Houston, TX, USA) with a pneumatic actuator model A36 (Valco Instruments) fitted with two PEEK loops of 1 μl (LC Packings). High Speed Switching Accessory (Valco Instruments) was used to increase helium flow through the pneumatic actuator in order to decrease the switching time of the valve. The HPLC pump was converted to deliver a microflow by interfacing the pump with a microflow processor (Accurate, LC Packings).

2.3. Operating conditions

The separation was performed on a microbore Nucleosil® C_{18} HD column, 150×1 mm I.D., with a 3-μm particle size and a 100-Å pore size (Macherey-Nagel, Düren, Germany) protected by an Inertsil® ODS3 guard column (2×0.8 mm I.D., particle size 5 μm, LC Packings) at ambient temperature. The mobile phase was composed of 20 m*M* diammonium phosphate buffer $(NH_4)_2HPO_4$ (adjusted to pH 7.0 with phosphoric acid 85% v/v)–acetonitrile (75:25, v/v).

Prior to use, the mobile phase was filtered under reduced pressure through a Durapore 0.22 μm filter (Millipore, Bedford, MA, USA) and was continuously degassed with helium. The flow-rate through the microbore column was maintained at 40 μl/min and the UV absorbance was measured at 210 nm with AUFS=0.2.

2.4. Preparation of analytical standards

Stock solutions of fluconazole and UK-54373 (1 mg/ml) were prepared by dissolving the appropriate amount of the drugs in methanol.

Six microdialysate standards containing 0.15, 0.25, 0.5, 1, 10 and 20 μg/ml of fluconazole were prepared by diluting stock solutions of fluconazole with phosphate buffer solution (0.01 *M* Na_2HPO_4 adjusted to pH 7.0 with phosphoric acid and isotonised with NaCl 8.288 g/l). Stock solutions and microdialysate standards were stored at 4 °C.

2.5. Assay validation

Validation of the assay procedure was carried out to establish intra- and inter-assay variability, linearity, and limits of detection and quantification over the calibration range.

Microdialysate calibration curves in the range of 0.15–20 μg/ml for fluconazole were constructed by plotting fluconazole peak area against the concentrations of the standards.

The precision was given by the RSD, while the accuracy was calculated as relative error (RE) of backcalculated concentrations from nominal concentrations. The limit of detection was the lowest concentration of fluconazole with a signal-to-noise ratio of 3. The lower limit of quantification was defined as the lowest standard on the calibration curve determined with an accuracy and a precision of 20% of the nominal value and a signal-to-noise ratio of 5. Intra- and inter-day accuracy and precision of the fluconazole assay for microdialysate samples were determined on 3 different days by triplicate analysis of six known concentrations, equally divided over the calibration curve. Statistical analysis was performed by one-way analysis of variance (ANOVA) with the statistical software SPSS 10.0.5. (SPSS, Chicago, IL, USA). A *P* value of 0.05 or less was considered significant.

2.6. In vivo microdialysis experiments

Hairless male rats (Iffa Credo, Saint-Germain, France), weighing between 280 and 320 g, were anesthetized with a ketamine–xylazine mixture (90:10 mg/kg) during the surgical procedure.

One day before the pharmacokinetic study a flexible microdialysis probe [CMA/20, 10-mm membrane length, and 20 000 molecular mass (M_r) cut-off] was implanted in the jugular vein with the use of a guide cannula. At 2 h before the start of the sampling period, a linear microdialysis probe (10 mm membrane length, 5000 molecular mass cut-off) was implanted in the dermis of the dorsal region of the rat [26].

Both probes were connected to a microinjection pump (CMA/100, Stockholm, Sweden) and perfused with 0.01 *M* Na_2HPO_4 at pH 7.0 isotonised with NaCl 8.288 g/l at a flow-rate of 0.5 μl/min. The retrodialysis marker, UK-54373, was introduced in the perfusate at a concentration of 10 μg/ml.

During the pharmacokinetic study, the rat was placed in a containment system (CMA/120) allowing the free movement and access to water and food.

Dialysates were directly collected into the injection loops and alternatively injected into the microbore HPLC system every 12 min during a period of 480 min after intravenous bolus of fluconazole at a dose of 10 mg/kg.

3. Results and discussion

3.1. Development of a microbore HPLC assay for fluconazole

With an on-line system, the choice of the pH value of the mobile phase is a critical point. In order to minimize the baseline disturbance, especially the injection front, when a microdialysate sample is injected on-line, the pH of the mobile phase was adjusted to a value of 7.0. It is the result of a compromise between the analytical considerations and the compatibility with the biological sample. At this pH value, fluconazole, a weak base with a pK_a

value of 2.03, was not ionized under the analytical conditions used resulting in a satisfactory retention. A primary problem of previously published methods for a reversed-phase HPLC separation of fluconazole and internal standard [17,18], is that there is a significant peak tailing presumably due to the interaction with residual silanol groups which are ionized at pH 4 or above [27].

Unlike Inagaki et al. [20] who used tris(hydroxymethyl)aminomethane added to the mobile phase, peak tailing was reduced by using a base deactivated octadecyl phase presenting a high density monomeric coating. The high density of the coating effectively shields the residual silanol groups from the analytes, resulting in a stationary phase with very low bleeding. The use of diammonium hydrogen phosphate in the mobile phase worked also towards decreasing the peak tailing. Indeed the cation ammonium is in competition with the analytes for the residual silanol groups. The reduction of peak tailing and the use of silica particles with 3-μm size reduce the peak width and improve the detection sensitivity.

With a conventional column (4.6 mm I.D.), the consumption of solvent and the associated cost are important especially for an on-line system. In the case of our on-line application, microbore HPLC reduces the solvent consumption and waste by a factor of 25.

In micro-LC techniques, the small internal diameter and slow flow-rate offer a significant signal enhancement in terms of reduced elution volume and noise. The direct consequence of this is that all dead-volume areas of the HPLC system must be carefully minimized. With microcolumns, most UV detectors available for microseparation techniques suffer from a loss in sensitivity due to the small cell volumes (10–100 nl; path length 50–320 μm) necessary to avoid peak broadening.

Initially developed by Chervet et al. [28], the UZ-View flow cell used here offers a path length of 30 mm for an inner volume of 540 nl. This flow cell design increases the sensitivity of the UV detection in accordance with the Beer–Lambert's law without increasing peak dispersion. For poorly absorbing compounds like fluconazole, the advantage of increased mass sensitivity and efficiency in microseparations coupled to the UZ-View flow cell provides a breakthrough in bioanalysis techniques.

3.2. Validation of HPLC assay

3.2.1. Retention times and selectivity

The mean retention times for fluconazole and UK-54737 and the RSD over 3 days were, respectively, 5.8 min (0.3%) and 10.2 min (0.3%). The retention factors (k) for fluconazole and UK-54373 obtained with acetonitrile–diammonium phosphate buffer (25:75, v/v) were 2.2 and 4.6, respectively. Under the chromatographic conditions, no additional peaks due to endogenous substances that could have interfered with the detection of the compounds of interest were observed. Representative chromatograms of blank microdialysate with and without UK-54373 as retrodialysis marker, and chromatograms of microdialysate obtained 1 h after intravenous bolus of fluconazole (10 mg/kg) are shown in Fig. 2.

3.2.2. Linearity, sensitivity

Good linearity was exhibited for fluconazole over the concentration range of 0.15–20 μg/ml. The coefficients of correlation for each individual calibration curve are summarized in Table 1. Because on-line injection of undiluted microdialysate samples requires a calibration curve over a wide concentration range, the linearity of the response detector over this range is relevant for fast and convenient quantitation without having to use a nonlinear regression software.

The inter- and intra-day RSDs of the slopes of five calibration curves were 0.64 and 0.71%, respectively. No significant difference was observed between intra- and inter-day values of the slopes and the intercepts (ANOVA, $P > 0.05$).

The limit of detection, defined as the lowest concentration with a signal-to-noise ratio of 3, was 0.1 μg/ml. The limit of quantification was set at 0.15 μg/ml. This is the lowest standard concentration satisfactorily meeting the acceptance criteria [29]. With 1 μl of sample volume, these values correspond, respectively, to 0.1 and 0.150 ng of fluconazole injected onto the column.

The use of the microcolumn in association with the UZ-View flow cell results in a higher mass sensitivity. A sufficient sensitivity was therefore obtained with a volume of sample 80-fold smaller

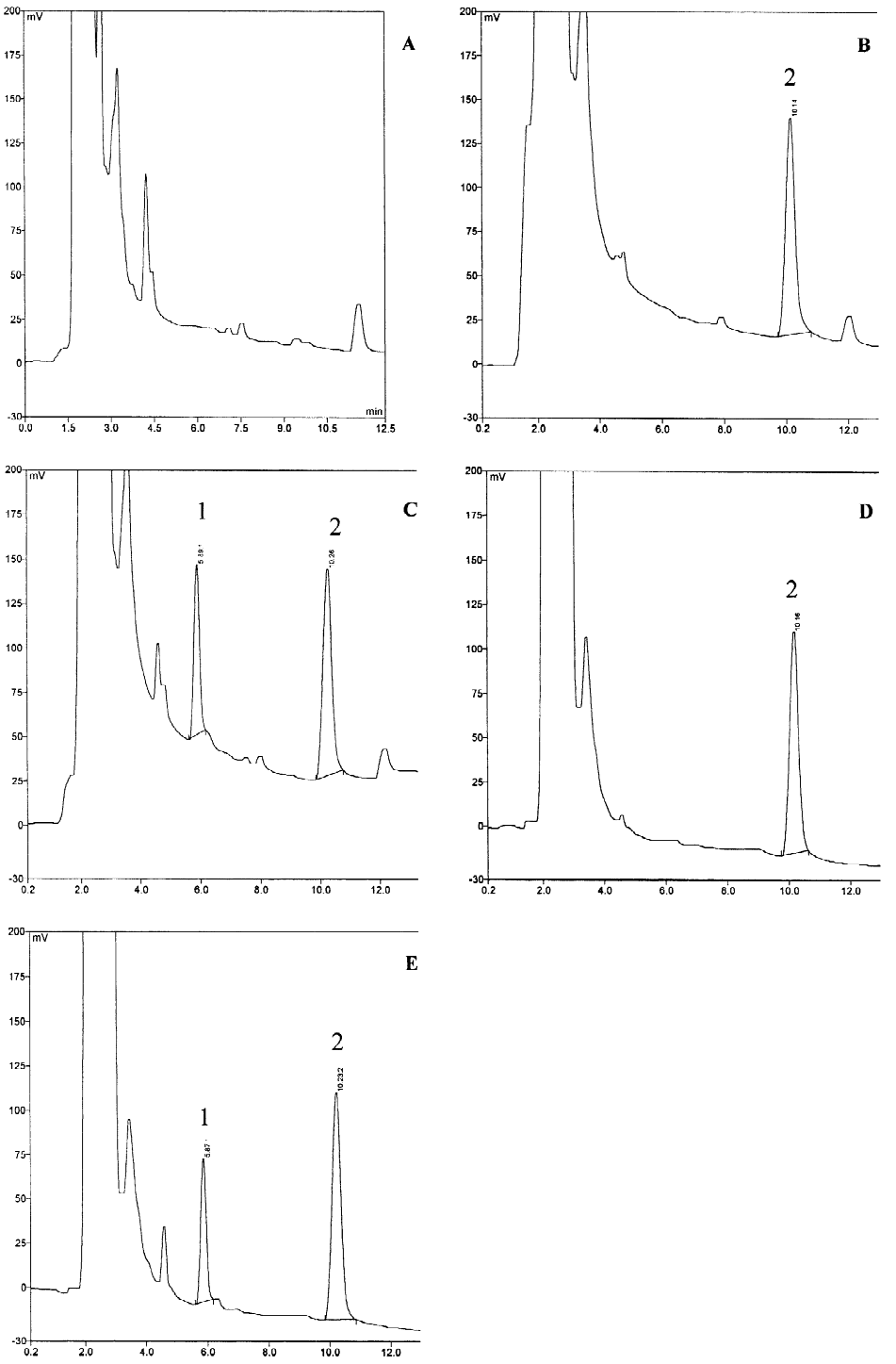

Fig. 2. Chromatograms of (A) blank blood dialysate, (B) blood dialysate before i.v. bolus injection, (C) blood dialysate 1 h after i.v. injection of a 10 mg/kg of fluconazole in the rat, (D) dermal dialysate before i.v. bolus injection, (E) dermal dialysate 1 h after i.v. bolus injection of a 10 mg/kg of fluconazole. (1 = fluconazole, 2 = UK-54373, retrodialysis marker).

Table 1
Individual and mean values for the slopes, intercepts and correlation coefficients of calibration curves in the fluconazole concentration range of 0.15–20 μg/ml in microdialysates on 3 days

Day	Slope	Intercept	r
Day 1	6.93	0.24	0.999
	6.83	0.48	0.999
	6.93	0.16	0.999
	6.86	0.30	0.999
	6.93	0.26	0.999
Mean±SD	6.90±0.05	0.29±0.12	
Day 2	6.95	0.36	0.999
	6.92	0.37	0.999
	6.96	0.30	0.999
	6.85	0.37	0.999
	6.95	0.31	0.999
Mean±SD	6.92±0.04	0.34±0.03	
Day 3	6.89	0.36	0.999
	6.96	0.30	0.999
	6.93	0.33	0.999
	6.92	0.29	0.999
	6.82	0.34	0.999
Mean±SD	6.91±0.05	0.32±0.03	

than with conventional HPLC [21] and 10-fold smaller than with a narrow-bore column [22].

3.2.3. Precision and accuracy

The intra- and inter-day precision of the fluconazole assay was good as indicated in Table 2 by the RSDs, which were always smaller than 2.7 and 5.4%, respectively.

The accuracy of the method was evaluated by calculating the RE. As shown in Table 2, the intra- and inter-day accuracy was 8.6% and 9.3%, respectively, at the limit of quantification.

3.3. On-line measurement of fluconazole in blood and dermal dialysates

Because microdialysis samples are protein-free and to avoid problems associated with the handling of small volume of samples, the microdialysis system was directly coupled with the microbore HPLC. Moreover, microdialysis being an in vivo sampling technique which does not withdraw body fluids, double-site sampling in the same animal is made feasible. To combine on-line analysis and double-site sampling, Heppert and Davies [24] configured a 10-port valve to collect dialysate samples of caffeine alternatively from the brain and jugular vein probes. In the present work, a microbore 10-port valve was configured to collect dialysate samples alternatively from the jugular vein and dermal probes to matched 1 μl loops. To minimize peak dispersion, a high-speed switching accessory was used to reduce the switching time of the valve. This system reduced the transit time of the valve by a factor of 10 to reach a

Table 2
Precision (repeatability) and accuracy of the HPLC assay for fluconazole in microdialysates

	Nominal concentration of fluconazole (μg/ml)	Calculated concentration of fluconazole (μg/ml, $n=5$)[a]	RSD (%)	RE (%)
Intra-day ($n=5$)	0.15	0.162±0.03	2.73	8.65
	0.25	0.234±0.005	2.34	−6.55
	0.50	0.49±0.01	2.17	−1.41
	1.00	1.007±0.005	0.53	0.76
	10.00	10.13±0.02	0.24	1.31
	20.00	19.93±0.01	0.06	−0.33
Inter-day ($n=3$)	0.15	0.164±0.02	6.32	9.34
	0.25	0.24±0.01	5.39	−4.05
	0.50	0.49±0.01	2.09	−2.05
	1.00	1.00±0.02	1.84	0.04
	10.00	10.13±0.08	0.75	1.28
	20.00	19.96±0.14	0.69	−0.21

SD, standard deviation; RSD, relative standard deviation; RE, relative error [(calculated−nominal)/nominal].
[a] Values expressed as mean±SD.

value of 28 ms with a helium pressure of 60 p.s.i. (1 p.s.i. = 6894.76 Pa)[1].

The applicability of the described method was demonstrated by measuring on-line the dialysate concentrations of fluconazole in blood and dermis following intravenous bolus injection of 10 mg/kg in the rat. The probes were calibrated in vivo by using the retrodialysis technique [10] with UK-54373 as retrodialysis marker. During the pharmacokinetic experiment, in vivo loss of UK-54373 was continuously monitored and was used to convert analyte microdialysate concentrations into extracellular concentrations [22].

Fig. 3 shows the unbound concentration–time profiles of fluconazole in blood and in dermis in a representative rat. This graph clearly illustrates that on-line microdialysis sampling allows the characterization of the unbound concentration–time curve with a discriminating resolution in the plasma and in the dermis. Moreover, coupled with the higher mass sensitivity of the microbore LC, temporal resolution of the microdialysis technique can be improved. Because the required volume of sample for a reliable quantification is smaller, the collection interval of microdialysates can be reduced from 20 to 12 min by using a column with 1 mm I.D. instead of 2.1 mm [22]. Therefore in a pharmacokinetic sense, the temporal resolution for fluconazole in a given tissue (blood or dermis) was 24 versus 40 min when a narrow-bore column was used with this double-site sampling set-up. A further development of the current method would consist in reducing the length of the column (100 or 70 mm) in order to decrease the chromatographic run time. A faster analysis would allow shorter collection intervals and thus a greater temporal resolution but the resolution of the separation may be decreased.

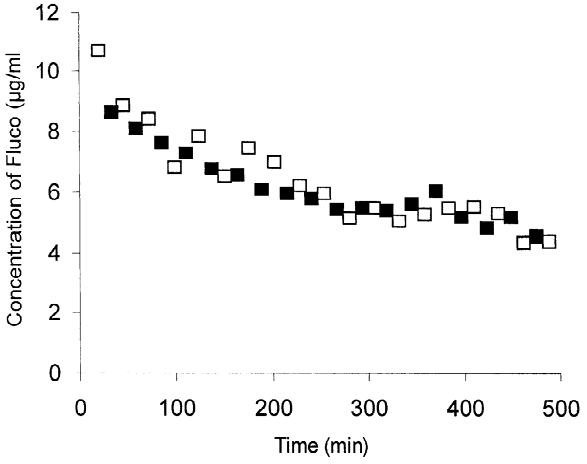

Fig. 3. Unbound concentration–time profile of fluconazole in blood (■) and in dermis (□) following i.v. bolus injection of 10 mg/kg to a representative rat.

[1]Technical note 412, Valco Instruments Inc.

4. Conclusions

To monitor simultaneously the unbound concentrations of fluconazole in blood and dermal tissues in the rat, a sensitive microbore HPLC system coupled directly to microdialysis sampling system was developed. A microbore 10-port valve equipped with a high-speed switching accessory was configured to alternatively collect the dialysate samples from the jugular vein and dermal probes. This on-line system allows to automate the analysis and to minimize the problems associated with the handling of small volume of samples. The dialysate samples were analyzed by using a microbore column with a high-density monomeric coating combined with a sensitive UV detector equipped with a flow cell presenting a path length of 30 mm. The resulting higher mass sensitivity achieved a limit of quantification of 0.15 μg/ml with an injected volume of 1 μl compatible with the microdialysis technique.

The method was selective, precise and accurate. The linearity was demonstrated over the concentration range of 0.15–20 μg/ml allowing pharmacokinetic studies by direct injection of undiluted microdialysates to be carried out.

In addition, the microbore technique allowed reduction of solvent consumption and waste by a factor of 25.

The developed method can be easily adapted and is now being used in our laboratory to quantify fluconazole in the dermis of the rat following topical delivery of fluconazole.

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