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Short communication

An ultra-fast LC method for the determination of iodiconazole in microdialysis samples and its application in the calibration of laboratory-made linear probes

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ABSTRACT

Iodiconazole is a very potent antifungal agent used to treat serious fungal infections. After transdermal administration, several factors affect the exposure of iodiconazole, resulting in large variability and demanding further elucidation of drug distribution. For determination of iodiconazole in dermal microdialysate, a new, efficient, reliable and robust ultra-fast liquid chromatography (UFLC™, Shimadzu) assay using UV detection at 230 nm has been developed and validated. Iodiconazole was separated on a Shimadzu Prominence UFLC™ C18 column (2.2 μm, 50 mm × 2.0 mm i.d.) using acetonitrile–0.025% triethylamine solution, adjusted to pH 3.6 with phosphoric acid (65:35, v/v), at a flow rate of 0.5 ml/min. The retention time was 1.37 min for iodiconazole and 1.78 min for the internal standard, an isomeric compound of iodiconazole. Intra- and inter-day precision ranged from 5.3% to 7.8% and 3.7% to 8.4%, respectively. The UFLC method was used to measure iodiconazole concentrations in microdialysis samples obtained during the calibration of laboratory-made linear probes. The validation and sample analysis results show that the method is precise, accurate and well suited to support the dermal microdialysis experiments.

1. Introduction

Triazole antifungal drugs play important roles in the treatment of fungal infection, which have good effect on both deep and superficial fungal infections [1]. One of these compounds is iodiconazole (1-(1H-1,2,4-triazole)-2-(2,4-diflurophenyl)-3-[*N*-methyl-*N*-(4-iodo-benzyl)amino]-2-propanol). Iodiconazole exhibits good antifungal activity against both systemic pathogenic fungi and dermatophytes. As for a topical antifungal agent, iodiconazole concentrations in the target tissue are considered most important for clinical efficacy. The microdialysis technique is a well established in vivo method for the monitoring of drug concentrations in the interstitial space of tissues, which is the major site of fungal infections [2]. For the analysis of iodiconazole in microdialysate, a sensitive method suitable for small sample volumes is required. Analytical combinations with tandem mass spectrometry were able to reduce the sample volume to 5–10 μl [3,4]. However, involatile phosphate can contaminate the ion source of tandem mass spectrometer. Hence, LC–MS/MS is inadequate for high-throughput, routine microdialysate investigations. A previously described high-performance liquid chromatography method for the quantification of iodiconazole in rat plasma is unsuitable for use on microdialysate because it includes liquid–liquid extraction of 200 μl samples and injection of 80 μl of the extract [5]. Our aim was to develop an alternative method that allows the analysis of the small sample volumes obtained by microdialysis with sensitivity comparable to that of existing methods, yet without the need of the expensive and technically demanding mass spectrometer. The use of small particles of stationary phase allowed UFLC to improve both peak capacity (due to higher efficiency) and speed of analysis (due to higher linear velocities) without compromising resolution. Thus, UFLC takes full advantage of chromatographic principles to run separations using columns packed with smaller particles (2.2 μm), with superior resolution and shorter analysis time. To this purpose we have developed and validated a high-throughput method using ultra-fast liquid chromatography for the determination of iodiconazole in microdialysate.

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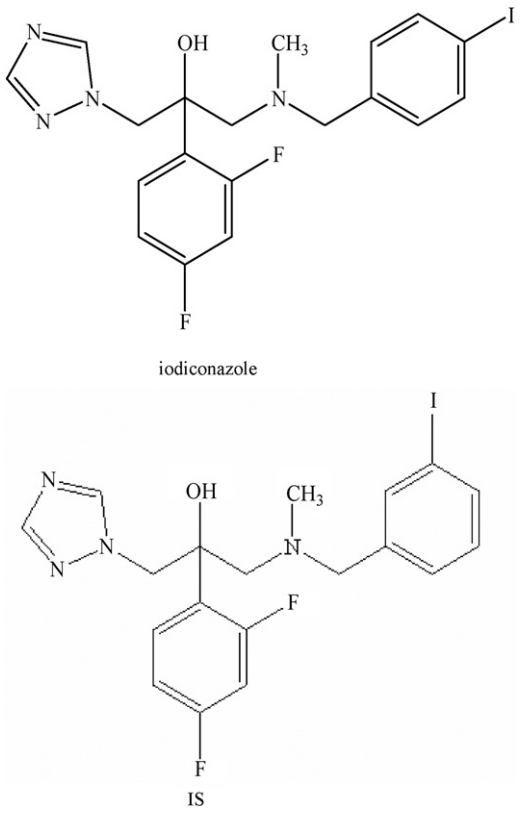

Fig. 1. Chemical structures of iodiconazole and IS.

2. Material and methods

2.1. Reagents and materials

Both iodiconazole and an isomeric compound of iodiconazole (the internal standard, IS) (Fig. 1, purity of both ≥98.0%) were confirmed by IR, MS and NMR spectroscopy and synthesized at the laboratory in the Pharmacy School of Second Military Medical University (Shanghai, China). Acetonitrile and methanol (all HPLC grade) were purchased from Merck Company (Darmstadt, Germany). All other reagents were of analytical grade. Isotonic phosphate buffer pH 7.4 (phosphate-buffered saline, PBS, 3.191 g/l Na_2HPO_4, 0.775 g/l NaH_2PO_4 and 5.58 g/l NaCl) was as an artificial extracellular fluid for perfusing the probe. Double-distilled water was used and 0.45 μm pore size filters (Millipore, MA) were used to filter the solutions. Microdialysis sampling was performed by using a BAS microdialyser (BAS Microdialysis, West Lafayette, USA). Microdialysis probes (Fig. 2) were prepared using single hollow cellulose fiber (200 μm inner diameter, i.d.; 220 μm outer diameter, o.d.; 5 kDa, DM-22 dialyzer, Eicom Ltd., Kyoto, Japan). The fiber was glued with cyanoacrylate glue (Cyberbond Co., Batavia, IL, USA) at both ends to a piece of quartz capillary tubing (98 μm i.d.; 165 μm o.d.; Polymicro Technologies Ltd., USA).

2.2. Chromatographic conditions

A Shimadzu Prominence UFLC™ system, equipped with a LC-20AD VP pump, a SIL-20AD VP automated sample injector, a thermostatted column compartment CTO-20AC VP, a SPD-20A UV detector operating wavelengths of 230 nm was used for iodiconazole analysis under UFLC conditions. Data were processed with LCsolution software (Shimadzu, Japan). IS (5 μl, 100 μg/ml in methanol) was added to microdialysate sample after each collection time.

Samples were analyzed on a Prominence UFLC™ C18 column (2.2 μm, 50 mm × 2.0 mm i.d., Shim-pack XR-ODS, Shimadzu); mobile phase consisted of acetonitrile–0.025% triethylamine solution adjusted to pH 3.6 with phosphoric acid (65:35, v/v); flow rate, 0.5 ml/min; column temperature, 35 °C; injection volume, 10 μl; analysis time, 2 min.

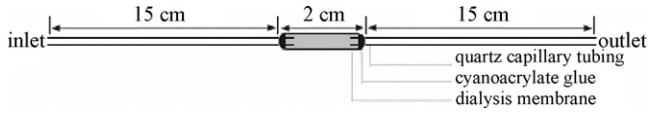

Fig. 2. Linear microdialysis probe.

2.3. Validation of UFLC method

A stock solution of 1 mg/ml of iodiconazole in methanol was used to prepare the calibration samples and quality control (QC) samples in PBS solution for analysis of the microdialysis samples. This stock solution was prepared weekly and the working solutions were diluted with PBS solution to appropriate concentrations daily. IS was prepared at a concentration of 100 μg/ml in methanol. Microdialysate calibration samples were prepared prior to each analytical run by mixing 5 μl of aqueous working solution with PBS solution, yielding iodiconazole concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, and 50.0 μg/ml. Calibration curves were established based on the peak area ratios of iodiconazole to IS against nominal iodiconazole concentrations using weighted ($w = 1/x$) linear regression analysis.

The specificity for iodiconazole was evaluated in blank samples collected by microdialysis from dermis tissue in rats that did not receive iodiconazole. Validation samples were prepared and analyzed to evaluate the intra-day and inter-day precision and accuracy, which were determined by quantitating five replicates at concentrations of the LLOQ, low, mid, and high concentration quality control samples (0.2, 0.4, 4.0, and 40.0 μg/ml) on the same day and five consecutive days. Mean, standard deviation, and relative standard deviation (R.S.D.) were calculated from QC values and used in the estimation of intra- and inter-day precision. Accuracy was assessed by comparison of the calculated mean concentrations to nominal concentrations.

The stability of iodiconazole was assessed in microdialysates collected from PBS solution (in vitro) and dermal tissue in rats (in vivo), reflecting situations likely to be encountered during actual sample collection, storage and analysis. Sample stability in microdialysates was investigated at 0.4, 4.0, and 40 μg/ml. Short-term stability was examined by analyzing samples at room temperature for 24 h. Long-term stability study was performed by analyzing samples stored for 2 months at −20 °C. For freeze–thaw stability study, the samples were left 1 h to thaw, and then refrozen for 24 h. This cycle was repeated three times and analysis was done after the third freeze–thaw cycle.

In addition, the microdialysis samples to which IS had been added were analyzed without prior sample preparation except for high-speed centrifugation at 10,000 × *g* for 10 min.

2.4. Application of the UFLC method

The UFLC method was used to measure iodiconazole concentrations in microdialysis samples obtained during the calibration of laboratory-made linear probes.

2.4.1. Effect of perfusion flow rate on recovery by dialysis and retrodialysis

Recovery by dialysis was studied by immersing the microdialysis probes (20 mm) in PBS (pH 7.4) containing 10 μg/ml iodiconazole (C_{sol}) as a dialysis medium. The probes were perfused with medium

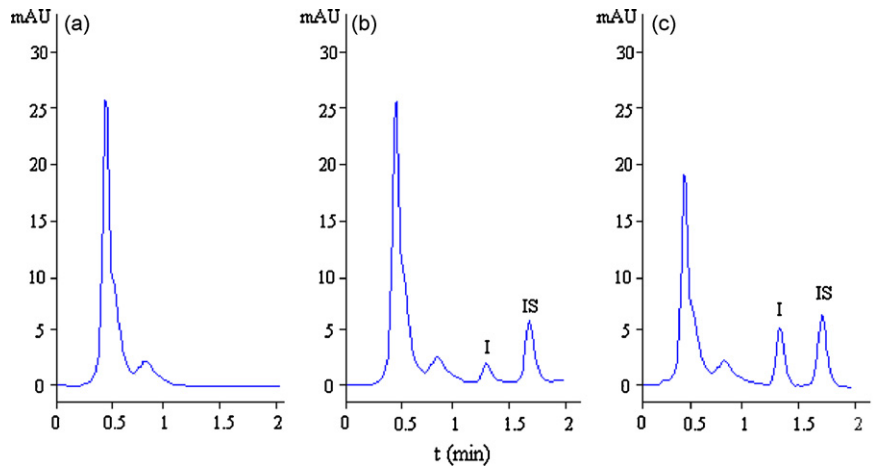

Fig. 3. Representative chromatograms of spiked microdialysates (dermis tissue) from a rat: (a) a blank microdialysate sample; (b) iodiconazole (I) at the LLOQ (0.2 μg/ml); and (c) iodiconazole 5.36 μg/ml. The retention times of iodiconazole and IS were 1.37 and 1.78 min, respectively.

PBS (pH 7.4) at five different flow rates (1, 2, 3, 4 and 5 μl/min). Four replicates of microdialysate sample (60 μl, C_{out}) were collected for each flow rate. The relative recovery by dialysis of iodiconazole was calculated as follows:

$$R(\%) = \frac{C_{out}}{C_{sol}} \times 100 \quad (1)$$

The same probes as used for the recovery by dialysis were also used for examining recovery by retrodialysis. The probes were immersed in PBS (pH 7.4) and perfused with PBS (pH 7.4) containing iodiconazole (10 μg/ml, C_{in}). Microdialysate samples (60 μl) were then collected and analyzed for iodiconazole (C_{out}). Flow rates and the number of replicates collected were the same as for recovery determination by dialysis. The relative recovery by retrodialysis of iodiconazole was calculated as follows:

$$R(\%) = \frac{(C_{in} - C_{out})}{C_{in}} \times 100 \quad (2)$$

2.4.2. Effect of concentration on recovery by dialysis and retrodialysis

For the determination of iodiconazole concentration influence on recoveries the flow rate was fixed at 3 μl/min and five different iodiconazole concentrations were used to determine the recovery by dialysis and retrodialysis: 2, 5, 8, 10, and 15 μg/ml. The systems were allowed to equilibrate for 1 h before the 20 min interval samples were collected up to 2 h. And four replicates of microdialysate samples (60 μl) were collected at each iodiconazole concentration.

2.4.3. Within-day stability of recovery

Within-day stability of recovery was studied by placing the probes (20 mm) in PBS (pH 7.4) containing iodiconazole (10 μg/ml), while the probes were perfused at 3 μl/min continuously for 8 h with PBS (pH 7.4). Microdialysate samples (60 μl) were collected over the 8 h study period, and recovery by dialysis was determined using Eq. (1).

2.4.4. In vivo calibration

Following the general surgical procedures and insertion of the microdialysis probe, a half-hour equilibration time was allowed for the tissue to recover with the probe perfused with drug-free PBS (pH 7.4). Perfusion fluid (3 μl/min) was then changed to PBS (pH 7.4) containing 10 μg/ml iodiconazole for in vivo calibration. Microdialysate samples (60 μl) were collected over 7 h. The in vivo recovery was expressed as the relative loss of iodiconazole from perfusion solution into subcutaneous extracellular fluid and calculated using Eq. (2).

3. Results

3.1. UFLC method development

Recently, an improvement in chromatographic performance has been achieved by the introduction of ultra-fast liquid chromatography (UFLC) [6]. The van Deemter equation indicates that, when the particle size decreases to less than 2.5 μm there is a significant gain in efficiency and that efficiency does not diminish at increased flow rates or linear velocities. UFLC system supports high-speed analysis through the following features: both low volume tubing and flow cell can decrease extra-column band spreading, 10 s ultra-fast injections with the Prominence SIL-20 autosampler, temperature capability up to 85 °C, fast data acquisition allows for better signal tracing. These features together with the use of a 2.2 μm particle size column, can shorten analysis time five- to sixfold, giving a considerable saving in both instrument and analyst time of value to those involved in high-throughput applications.

An isomeric compound of iodiconazole was selected as the internal standard [5], because its maximum absorbance was also 230 nm and had a suitable retention time. The mobile phase pH had a significant effect on the retention time of iodiconazole and IS, with shorter retention times being obtained at lower pH values due to the addition of phosphoric acid. In this study, our mobile phase was adjusted to pH 3.6 with phosphoric acid yielded retention times of only 1.37 min for iodiconazole and 1.78 min for IS which allows a

Table 1
Intra-run and inter-run precision and accuracy of iodiconazole in microdialysate (intra-run: $n = 5$; inter-run: $n = 5$ per day, 5 days).

Concentration (μg/ml)		Precision R.S.D. (%)	Accuracy (%)
Spiked	Observed (mean ± S.D.)		
Intra-run			
0.2 (LLOQ)	0.186 ± 0.014	4.2	98.4
0.4 (low)	0.397 ± 0.042	6.1	94.5
4.0 (middle)	3.80 ± 0.73	7.8	97.3
40.0 (high)	40.41 ± 2.15	5.3	103.1
Inter-run			
0.2 (LLOQ)	0.191 ± 0.005	5.8	96.7
0.4 (low)	0.380 ± 0.025	8.4	91.5
4.0 (middle)	4.05 ± 0.76	3.7	104.8
40.0 (high)	40.32 ± 3.57	6.5	108.6

Table 2
Results of perfusion flow rates and concentrations on the in vitro recovery determined by dialysis and by retrodialysis of iodiconazole from the microdialysis probe (means ± S.D. for three probes, $n = 3$ for each probe).

	Flow rate (μl/min)					Concentration (μg/ml)				
	1	2	3	4	5	2	5	8	10	15
Dialysis (%)	78.1 ± 4.5	67.7 ± 3.7	47.8 ± 9.1	34.1 ± 8.7	28.1 ± 7.9	47.8 ± 7.1	48.2 ± 5.8	48.1 ± 6.4	47.7 ± 6.8	47.8 ± 8.9
Retrodialysis (%)	82.4 ± 6.9	70.3 ± 5.4	48.1 ± 7.7	33.7 ± 5.2	27.9 ± 6.3	48.3 ± 6.4	47.5 ± 7.2	48.2 ± 6.3	48.4 ± 4.6	47.7 ± 5.8

high sample throughput. Major advantages of UFLC are mostly economical, i.e., low consumption of solvents, smaller injection volume and five times higher sample throughput.

3.2. UFLC method validation

Typical chromatograms of iodiconazole are shown in Fig. 3. Each analysis of microdialysate was completed within 2 min. Under the UFLC conditions described iodiconazole and IS gave peaks of good symmetry and were well separated from matrix compounds. A weighted linear regression was used to perform standard calibration. The mean calibration equation was $y = 0.7798$ (R.S.D. = 4.1%, $n = 5$) $x - 0.0173$ (R.S.D. = 2.9%, $n = 5$). Calibration curves showed excellent linearity in the range of 0.2–50.0 μg/ml with correlation coefficients consistently greater than 0.999. The limit of detection (LOD) based on a signal-to-noise ratio of 3 was 0.02 μg/ml, while the lower limit of quantification (LLOQ) used in the construction of the calibration curves was 0.2 μg/ml with a signal-to-noise ratio of 10. Table 1 shows a summary of intra- and inter-day precision and accuracy of QC samples. Intra- and inter-day R.S.D. ranged from 3.7% to 8.4% and accuracy ranged from 91.5% to 108.6%. Iodiconazole was found to be stable in microdialysate samples (in vitro and in vivo) for at least 24 h when stored at room temperature and for 2 months when stored at −20 °C (R.S.D. < 15%). The stock solutions of IS were stable for at least 12 h when stored at room temperature and for 2 months when stored at −20 °C (R.S.D. < 15%).

3.3. Results of the in vitro and in vivo microdialysis experiments

In vitro recovery of iodiconazole from the microdialysis probe determined by dialysis and by retrodialysis was compared at each flow rate and concentration (Table 2). Iodiconazole recovery decreased as the flow rate increased and was independent of concentration over the concentration range investigated. An ANOVA analysis of the results did not reveal any difference in the recoveries obtained for all concentrations tested for two methods ($P > 0.1$). A flow rate of 3 μl/min was selected for both in vitro and in vivo studies based on acceptable sampling duration and volume. The performance of the microdialysis system was stable over an 8 h study, resulting in a mean in vitro recovery of 47.6 ± 1.3%. In the present study, in vivo recovery of iodiconazole was 33.6 ± 5.4% and was stable over the 7 h study period.

4. Discussion

A 50 mm column submitted to an isocratic flow rate of 0.5 ml/min for 2 min was used to obtain the chromatograms. The very narrow chromatographic peaks generated by UFLC, resulted in an increase in the chromatographic efficiency and sensitivity. A sensitive, selective and rapid UFLC method for the determination of iodiconazole in microdialysate is described for the first time. All parameters met the criteria set in international guideline for bioanalytical methods. The overall results from the in vitro study showed that the recovery of iodiconazole from the microdialysis probe was about 48%. The in vivo recovery of iodiconazole from subcutaneous extracellular fluid was 34% by delivering iodiconazole into subcutaneous tissue. In conclusion, a rapid and reliable UFLC assay was established and validated for the determination of iodiconazole concentrations in microdialysate samples.

References

[1] P.L. Shao, L.M. Huang, P.R. Hsueh, Recent advances and challenges in the treatment of invasive fungal infections, Int. J. Antimicrob. Agents 30 (2007) 487–495.
[2] D.M. Saunte, F. Simmel, N. Frimodt-Moller, L.B. Stolle, E.L. Svejgaard, M. Haedersdal, C.K. Loft, M.C. Arendrup, In vivo efficacy and pharmacokinetics of voriconazole in an animal model of dermatophytosis, Antimicrob. Agents Chemother. 51 (2007) 3317–3321.
[3] R.N. Tettey-Amlalo, I. Kanfer, Rapid UPLC-MS/MS method for the determination of ketoprofen in human dermal microdialysis samples, J. Pharm. Biomed. Anal. 50 (2009) 580–586.
[4] S.H. Gao, X. Tao, L.N. Sun, C.Q. Sheng, W.N. Zhang, Y.L. Yun, J.X. Lia, H.J. Miao, W.S. Chen, An liquid chromatography–tandem mass spectrometry assay for determination of trace amount of new antifungal drug iodiconazole in human plasma, J. Chromatogr. B 877 (2009) 382–386.
[5] J. Wen, G.R. Fan, Z.Y. Hong, Y.F. Chai, X.P. Yin, Y.T. Wu, C.Q. Sheng, W.N. Zhang, High performance liquid chromatographic determination of a new antifungal compound. ADKZ in rat plasma, J. Pharm. Biomed. Anal. 43 (2007) 655–658.
[6] N. Sun, G.C. Lu, M. Lin, G.R. Fan, Y.T. Wu, High-throughput quantification of a novel thiazolidinedione MCC-555 in rat plasma by ultra-fast liquid chromatography and its application in pharmacokinetic studies, Talanta 78 (2009) 506–512.