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Determination of a new antifungal agent, voriconazole, by multidimensional high-performance liquid chromatography with direct plasma injection onto a size-exclusion column

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Abstract

A fully automated method has been developed for the analysis of a new antifungal agent, voriconazole, in human plasma. Multidimensional chromatography was used with size-exclusion chromatography as the first step to separate plasma protein from the drug and internal standard which were then trapped on a precolumn of pellicular ODS. A reversed-phase column, Spherisorb ODS2, then separated drug and internal standard from one another and from remaining plasma components. With an injection of 0.56 ml plasma the limit of quantitation of the method was 5 ng/ml.

Keywords: Voriconazole

1. Introduction

Voriconazole (I; Fig. 1) is a novel broad spectrum antifungal drug exhibiting cidality against *Aspergillus* species. The initial pharmacokinetic studies in man were carried out with a method involving solvent extraction, back extraction and re-extraction for sample preparation prior to HPLC (personal communication, M.W.H. Clark) This method was tedious and time-consuming and an automated and higher throughput method was required for samples from clinical trials. A bioassay was also available but was not sufficiently sensitive for pharmacokinetic studies and the specificity for unchanged drug had not been evaluated. The method described here has been developed with direct injection of plasma on a size-exclusion column as the initial step.

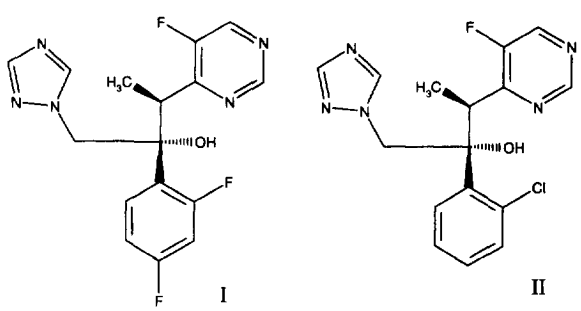

Fig. 1. Structures of voriconazole (I) and internal standard, UK-115 794 (II).

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2. Experimental

2.1. Chemicals

Voriconazole (I, UK-109 496) and internal standard (II, UK-115 794, Fig. 1) were obtained from Pfizer Central Research (Sandwich, UK). Acetonitrile and methanol, both Super Purity Solvent grade, were obtained from Romil Chemicals (Loughborough, UK). N,N,N′,N′-Tetramethylethylenediamine (TEMED), GPR grade, phosphoric acid, 85%, GPR grade, ammonia solution sp. gr. 0.88, Analar, and ammonium dihydrogenphosphate, Analar, were obtained from Merck (BDH) (Lutterworth, UK). Ultrapurified water was provided by a Milli-Q water purification unit (Millipore, Watford, UK).

Coomassie Plus protein reagent and bovine serum albumin standard, 2 mg/ml, were obtained from Pierce and Warriner (Chester, UK).

2.2. Chromatography

The HPLC system (Fig. 2) was constructed from the following components: three Shimadzu LC-6A pumps, a Shimadzu SPD-6A UV detector set at 255 nm (Dyson Instruments, Hetton, UK), a Gilson 232 autosampler (Anachem, Luton, UK) with a 1-ml sample loop, a Rheodyne 7010 switching valve (on the Gilson autosampler) and an electrically actuated Valco WE-C6WPK switching valve (Thames chromatography, Maidenhead, UK). The switching valves were controlled from the autosampler. Data collection was carried out by a Multichrom 2.1 chromatography data system (VG Data Systems, Fisons Instruments, Loughborough, UK). Autosampler vials were glass, 2 ml with screw caps and a natural rubber/PTFE seal (Chromacol, Welwyn Garden City, UK).

Column 1 was a Pharmacia HR 10/10 fast desalting column packed with Sephadex G-25 superfine (Pharmacia Biotech, St Albans, UK) to give a column of 100×10 mm I.D. with a mobile phase of ammonium phosphate buffer, 0.02 *M*, pH 6.5 at a flow-rate of 1.0 ml/min. Column 2 was a Chrompack precolumn 10×2.1 mm I.D. (Chrompack, London, UK) dry packed with Whatman pellicular ODS, 37.5 μm (Whatman International, Maidstone, UK) with 20 μm stainless steel screens and was flushed with methanol at a flow-rate of 0.8 ml/min when it was not in line with column 1. The analytical column, column 3, was Spherisorb ODS2, 5 μm, 250×4.6 mm I.D. (Hichrom, Reading, UK) with a mobile phase of acetonitrile–TEMED phosphate buffer 0.1 *M*, pH 7.0 (42:58) and a flow-rate of 0.8 ml/min. The TEMED buffer was prepared by adding phosphoric acid, 85%, to TEMED solution, 0.1 *M*, to adjust the pH to 7.0. The analytical column was protected by a guard column, 10×3.2 mm I.D. packed with the same packing (Hichrom, Reading, UK). All chromatography was performed at room temperature.

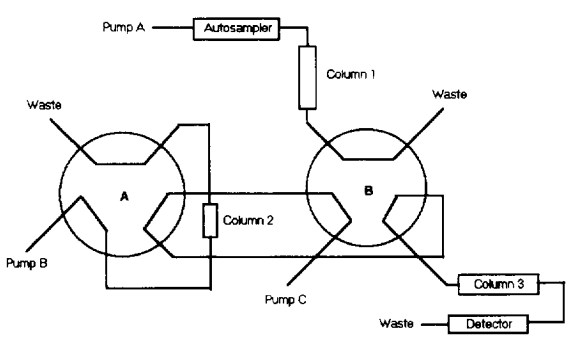

Fig. 2. Schematic diagram of the multidimensional chromatographic system with the valves in the "off" position.

2.3. Column switching

Plasma samples were injected onto column 1 and plasma proteins separated from drug and internal standard by size-exclusion chromatography. After the proteins had eluted the eluate from column 1 was diverted to column 2 where I and II were trapped and concentrated. I and II were transferred to column 3 and data acquisition started. The valve switching sequence is given in Table 1. Once the compounds from column 2 had been transferred to column 3 the next plasma sample could be injected onto column 1.

Before processing each batch of samples the correct valve timing sequence was determined by connecting the Sephadex column directly to the UV

Table 1
Column switching events

Time (min)	Event	Valve position	
		Valve A	Valve B
0	Inject plasma on column 1	Off	Off
4	Trap I and II on column 2	On	On
12	Transfer I and II to column 3 and start data acquisition	On	Off
13	Re-equilibrate column 2 and chromatography of I and II on column 3	Off	Off
29	End data acquisition	Off	Off

detector and 0.7 ml of a solution of I and II, both 10 μg/ml in ammonium phosphate buffer 0.02 *M*, pH 6.5, was injected.

2.4. Sample preparation and standards

Stock solutions of I and II were prepared in methanol and stored at 5°C. The stock solution of I was diluted with TEMED phosphate buffer, 0.05 *M*, pH 7.0, to give concentrations of 5 and 100 μg/ml before addition to plasma. Calibration standards were prepared by mixing aliquots of these diluted solutions with 0.7 ml control human plasma to give concentrations over the range 10 to 3000 ng/ml.

Control human plasma containing known concentrations of I (40, 600 and 1900 ng/ml, quality assurance samples) were stored at −20°C and analysed in duplicate with every analytical run to judge assay performance. In each batch calibration standards and quality assurance samples were distributed at random among the samples from clinical trials.

Plasma samples (clinical or study samples, quality assurance samples and standards), 0.7 ml, were mixed with 0.3 ml of a solution of II (4.5 μg/ml, diluted from the stock solution with ammonium phosphate buffer, 0.02 *M*, pH 6.5) in autosampler vials and 0.8 ml (corresponding to 0.56 ml plasma) was injected on column 1.

Linear calibration curves were fitted by weighted $(1/y^2)$ least squares regression analysis of the peak-height ratio of I/II vs. the concentration of I in the calibration standards. Concentrations of I in clinical trial samples and quality control samples were estimated from the peak-height ratio of I/II and the calibration curve parameters obtained above.

2.5. Chromatography on the Sephadex column

Blank plasma and plasma containing I and II (20 μg/ml of each compound) were injected onto the Sephadex column as described in Section 2.4 and the eluate was monitored at 255 nm. Aliquots (1 ml) of the eluate were collected and analysed for protein using a Coomassie blue reagent (0.1 ml eluate and 3 ml reagent) to produce a blue colour for which the absorbance was measured at 595 nm. A calibration curve was prepared with bovine serum albumin over the range 50 to 1600 μg/ml.

2.6. Recovery

Compound I, 470 ng/ml and II, 417 ng/ml in control human plasma were injected into the HPLC system and the peak areas were compared with the same amount of I and II in ammonium phosphate buffer, 0.02 *M*, pH 6.5, injected directly into the analytical column.

2.7. Stability of I

Plasma samples from clinical trials were pooled and then frozen and thawed three times with an aliquot analysed in duplicate each time. Additional pooled samples were also stored at about −20°C and aliquots analysed at various times up to 14 months.

2.8. Interference from other drugs

Several drugs which may be co-administered with I were tested by HPLC on the analytical column. These drugs were paracetamol, rifampicin, prednisolone, dideoxyinosine and azidothymidine.

3. Results and discussion

3.1. Chromatography

Direct injection of plasma with precolumns has been extensively used for sample clean-up [1,2]. However these precolumns have a limited capacity for human plasma (samples of about 0.1 ml and a total of 15 ml plasma for each precolumn has been suggested [1]). They were considered unsuitable for I as a minimum of 0.5 ml plasma was required to achieve a sensitivity on 10 ng/ml and batches of at least 60 samples would be analysed.

Restricted access columns [3–11] are available for direct plasma injection although at the time this assay was developed (1991) we were not aware of any commercially available precolumns packed with restricted access materials which would allow the injection of 0.5 ml plasma. The main columns available at this time were Pinkerton analytical columns which were designed for injections of 10–20 μl of plasma. Attempts to inject 0.5-ml plasma samples onto a Pinkerton GFF ISRP guard cartridge (10×3 mm) failed due to blockage of the column after a few injections. In 1996 a restricted access alkyl-diol silica, developed by prof. K.-S. Boos [12–15], became available commercially as LiChrospher PR-18 ADS. We have recently tested a precolumn (25×4 mm) packed with 25 μm particles of this material with injections of 0.5 ml plasma with promising results suggesting a sensitivity of 10 ng/ml should be achievable.

A commercially available column of Sephadex G25 was found to give a good separation of I and II from plasma protein with sample volumes up to 1 ml plasma. Compounds I and II are eluted in one peak when injected in buffer (Fig. 3A) or in plasma (Fig. 3C). A chromatogram of blank plasma (Fig. 3B) with the analysis of protein with Coomassie blue showed (Fig. 3B) that plasma proteins were eluted prior to I and II. Sephadex has the interesting property of interacting with aromatic compounds [16] and this interaction enhances the separation of I and II from plasma proteins and allows the injection of fairly large volumes of plasma on this column. The peak observed after I and II in blank plasma is due to aromatic compounds retained by the Sephadex G25.

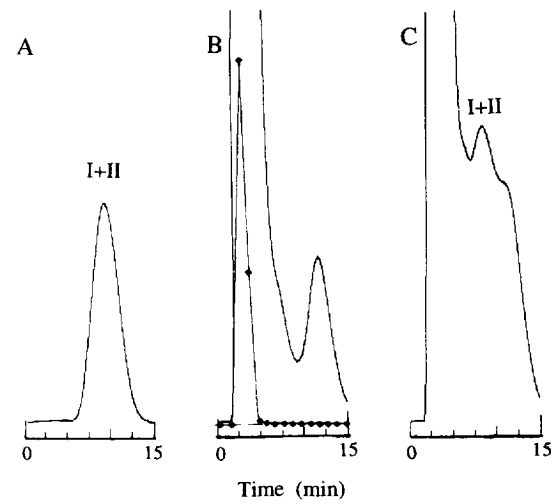

Fig. 3. Chromatograms on the Sephadex G25 column, monitored at 255 nm, of (A) I and II, 16 μg each, in ammonium phosphate, 0.02 *M*, pH 6.5, (B) plasma blank with protein determined by Coomassie blue ♦-♦, (C) plasma containing I and II, 20 μg/ml of each compound.

The reproducibility of the elution volume for I and II from the Sephadex G25 column was investigated by injecting 0.8 ml of a solution of I and II (each compound 16 μg/ml) in ammonium phosphate buffer, 0.02 *M*, pH 6.5. For I (n=48) retention time was 9.13 min (R.S.D. 0.71%), peak height was 119.7 mV (R.S.D. 1.46%) and peak width at half height was 114 s (R.S.D. 2.31%). For II (n=49) retention time was 10.77 min (R.S.D. 0.48%), peak height was 99.7 mV (R.S.D. 1.99%) and peak width at half height was 114 s (R.S.D. 2.76%).

Different Sephadex columns gave reproducible chromatography of I and II. For example during the analysis of plasma samples from a clinical trial 31 Sephadex columns were packed over a period of four months. The mean valve switching times for transfer of I and II from the Sephadex column to the

precolumn were 5.7 min (R.S.D. 7.75%) to 14.4 min (R.S.D. 3.81%) after injection. The increased R.S.D. for the initial switch was due to switching some samples 0.5 min earlier to trap the N-oxide metabolite of voriconazole which elutes from the Sephadex column slightly earlier than I.

The retention times of I and II on column 3 were very consistent when plasma samples were injected into the whole system. For example during an overnight run of 36 plasma samples the mean retention times were 11.51 min (R.S.D. 0.77%) for I and 13.55 min (R.S.D. 0.82%) for II.

There have been few reports of size-exclusion chromatography as the initial step for the automated analysis of drugs in plasma samples [17–22]. Columns of TSK-GEL SW (silica based) and TSK-GEL PW (hydrophilic porous polymer), Tosohaas, have been used with small plasma volumes (5 μl) [22] and with supernatants from pig tissue homogenates [20]. A Zorbax GF250 column was used for plasma samples up to 0.2 ml [17]. Interestingly a Sephadex G50 column has been used for on-line sample pretreatment [21] although plasma proteins in 1 ml plasma were first precipitated with trichloroacetic acid and the supernatant applied to the column.

Several types of precolumn were tested and the best results obtained with pellicular C_{18} particles. Smaller (5 μm or 10 μm) porous C_{18} particles were rejected as they blocked more frequently. Stainless steel screens in place of frits were also essential to reduce blockages.

Typical second dimension chromatograms of blank plasma, I and II added to plasma and a clinical study sample after p.o. administration of I are shown in Fig. 4A–C, respectively. Fig. 4A shows that no endogenous compounds from plasma gave significant peaks near the retention time of I or II.

Sephadex G25 is a fairly fragile gel and requires a maximum pressure limit of about 180 p.s.i. (1 p.s.i.= 6894.76 Pa) for this column. If this limit is reached the pump is set to switch off and the autosampler is automatically stopped to prevent loss of samples. These columns were replaced after about 100 samples and are easily repacked with about 2 g Sephadex G25. The precolumn and guard columns were replaced routinely every 100 samples to reduce the chance of the system blocking as this was the main problem experienced with direct plasma injection.

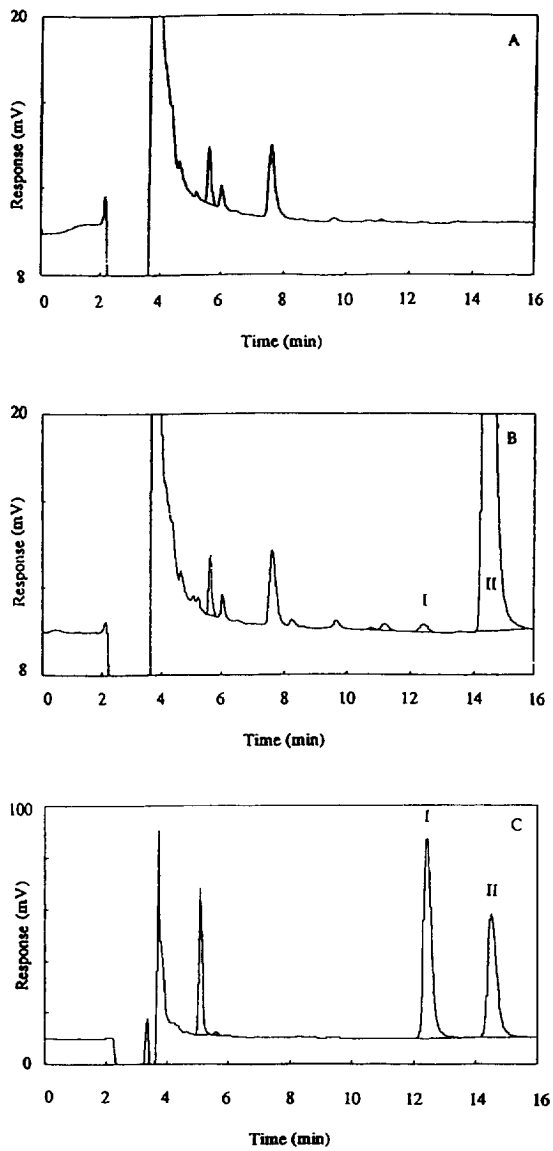

Fig. 4. Second dimension chromatograms: (A) human plasma blank, (B) I, 10 ng/ml, in human plasma, II internal standard, (C) I, 2409 ng/ml, in human plasma, II internal standard.

3.2. Recovery

The overall recovery of I and II after injection of plasma into the complete HPLC system was 97% (n=4, range 93–99% R.S.D. 2.74%) and 94% (n=4, range 91–98% R.S.D. 3.41%), respectively.

Plasma protein binding has little effect on the

Table 2
Intra-day accuracy and precision for I added to human plasma

Concentration added (ng/ml)	Concentration found (mean, $n=10$) (ng/ml)	Accuracy (error, %)	Precision (R.S.D., %)
2	2.32	16.1	22.8
5	4.8	−4.1	8.4
10	9.82	−1.8	7.2
20	20.0	0	5.6
600	580	−3.3	1.0
3000	2837	−4.9	0.8

recovery of drugs from plasma in this HPLC system as size-exclusion chromatography provides an efficient separation of bound drug from plasma protein. Plasma protein binding for I was 58% and good recovery for an antifungal drug with 99% protein binding has been obtained. In this respect size-exclusion chromatography is more efficient than dialysis for the separation of drugs from plasma in an automated system.

3.3. Calibration, precision and accuracy

A calibration range of 10 to 3000 ng/ml was used as this was appropriate for the plasma concentrations encountered in clinical studies. The calibration was linear over this range. For 10 curves prepared on separate days the mean slope was 0.000666±0.0000272 S.D. with a y-intercept of −0.00076±0.000638 S.D. The mean correlation coefficient was 0.9985.

The intra-day accuracy and precision was determined at 6 concentrations of I and the results (Table 2) were satisfactory except at 2 ng/ml where the precision exceeded 20%. The inter-day accuracy and precision were calculated from the results for quality control samples which were analysed in duplicate on five separate days. The results (Table 3) showed that the error in both accuracy and precision is less than 10%.

A conference report [23] recommended that at the limit of quantitation (LOQ) the precision should be 20% or less. As 5 ng/ml is the lowest concentration we have validated which has a precision of less than 20% we have taken this value as the LOQ. At 2 ng/ml the signal to noise ratio (peak height to peak-to-peak noise) is about 8. However small peaks of endogenous compounds occur close to the retention time of voriconazole and these vary with plasma sample. These peaks preclude the calculation of a limit of detection in terms of signal-to-noise ratio.

Peak-height ratio was used instead of peak-area ratio as this gave improved precision and accuracy for low concentrations of voriconazole in the validation. For example at a concentration of 5 ng/ml ($n=10$) the precision (R.S.D.) was 8.4% for peak height and 17.2% for peak area and the accuracy was −4.1% for peak height and 14.0% for peak area. This improved reliability with peak height has been reported by Mueller and Eitel [24]. This increased variation with peak area may be caused by problems in establishing peak start and peak end by the

Table 3
Inter-day accuracy and precision for I added to plasma and analysed in duplicate on five separate days

Concentration added (ng/ml)	Concentration found (mean, $n=10$) (ng/ml)	Accuracy (error, %)	Precision (R.S.D., %)
40	37.3	−6.8	5.9
600	562.5	−6.3	5.0
1900	1865	−1.9	5.7

Table 4
Stability of I in human plasma at −25°C

Time (months)	n	Voriconazole (mean±S.D.) (ng/ml)	Change from time 0 (%)
0	3	71.0±0.46	–
1	6	74.3±7.65	4.7
3	6	75.1±1.97	5.8
7	6	76.2±1.92	7.3
14	6	75.0±1.53	5.6

chromatography data system for low concentrations of drug due to variations in baseline between chromatograms of plasma extracts. This will affect peak area more than peak height.

3.4. Stability

Compound I in pooled plasma samples was stable to two freeze–thaw cycles with a change in concentration of −1.1% from the initial value of 75.5 ng/ml after one cycle and a −1.5% change after two cycles. I was also stable in human plasma at −25°C (Table 4) with no significant loss after 14 months.

3.5. Interference from other drugs

HPLC on column 3 of drugs which may be co-administered showed (Table 5) that none would interfere with the determination of I. When these drugs were injected onto the whole system only rifampicin and prednisolone appeared on column 3. As all the drugs were shown to elute from the Sephadex column during the period it was connected to the precolumn three drugs, paracetamol, dideoxyinosine and azidothymidine, were lost as they were not trapped on the precolumn.

Table 5
Interference from other drugs

Compound	Relative retention time	
	Column 3	Whole system
Voriconazole	1.0	1.0
Paracetamol	0.32	[a]
Rifampicin	0.88	0.90
Prednisolone	0.43	0.45
Dideoxyinosine	0.31	[a]
Azidothymidine	0.31	[a]

[a] These compounds were not retained by the precolumn.

4. Conclusions

A fully automated method has been developed for the analysis of voriconazole (I) with direct injection of plasma onto a Sephadex G25 size-exclusion column as the initial step. This method has been used for the analysis of over 3000 plasma samples from clinical trials and the only problem encountered with the method has been occasional blockages.

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