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Fast, fully automated analysis of voriconazole from serum by LC–LC–ESI-MS–MS with parallel column-switching technique

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Abstract

Voriconazole is a novel broad-spectrum antifungal agent. We developed an on-line LC–LC–MS–MS method for fully automated and direct analysis of voriconazole in raw human serum. After injection of human serum size-selective sample fractionation and analyte extraction was achieved using an extraction column (25 mm × 4 mm) packed with a restricted access material (RAM, LiChrospher® ADS C_8, 25 μm). On-line transfer of voriconazole from the extraction column was followed by chromatography separation on a C_{18} column. Detection was done by ESI-MS–MS. The total analysis time was 13 min, managed by parallel extraction and chromatographic separation. This LC–MS assay was fully validated. The lower limit of quantification was 0.05 μg/ml. The automated inline extraction of voriconazole described here eliminates the need for difficult and time-consuming sample pre-treatment. Other advantages of the new method are that only a small quantity (5 μl) of serum is needed and that the method is very specific.
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Keywords: Voriconazole; Column-switching; LC–LC–ESI-MS–MS; Serum; On-line sample preparation

1. Introduction

Voriconazole ((2*R*,3*S*)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1,2,4-triazol-1-yl)-2-butan-2-ol) (UK-109,496) (chemical structure in Fig. 1A) is a novel broad spectrum antifungal agent. It is a derivative of fluconazole. Voriconazole is an antifungal triazole with an expanded spectrum of activity against a variety of yeasts and filamentous fungi [1]. As with all triazole antifungal agents, voriconazole works principally by inhibition of cytochrome P450 dependent lanosterol 14-α-demethylase ($P450_{LDM}$) [1].

A number of clinical reports about voriconazole pharmacokinetics have been published [2–11], as well as numerous data obtained in in vitro investigations on the pharmacokinetic properties and activity of voriconazole [12–25].

Various analytical methods have been used in these studies. To the authors' best knowledge, so far, five HPLC or LC–MS methods had been published for the determination of voriconazole in serum [3,26–29] and one LC–MS method for the determination of voriconazole in aqueous humor [30].

Gage and Stopher describe two different methods. The first [29] involves column-switching of three columns. (i) First, they inject plasma directly into a size-exclusion column, (ii) then they switch to a concentration column, and (iii) finally to the analytical reversed-phase column. Their injection volume is 0.8 ml with 0.56 ml plasma and 0.24 ml internal standard. One run takes approximately 15 min in the parallel mode. Detection is done by UV. Their second method [26] is a very simple method for the determination of voriconazole from plasma. This method utilises protein precipitation with acetonitrile as the only sample preparation involved prior to reverse phase HPLC. The sample consists of 0.5 ml plasma and no internal standard. Detection is also done by UV (255 nm). Chromatographic separation requires 10 min. Pennick [27] use solid-phase extraction technology and need 0.5 ml plasma and an internal standard. They reached a limit

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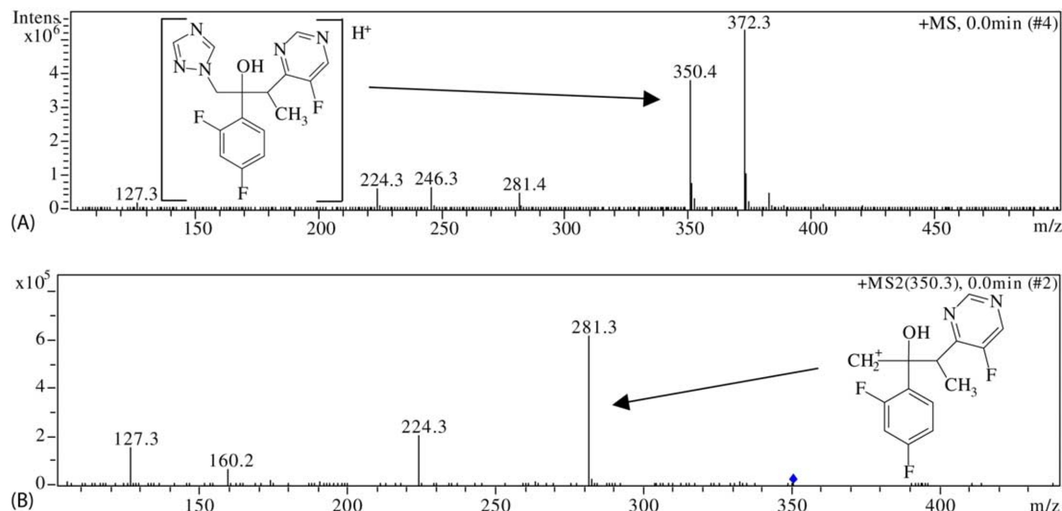

Fig. 1. (A) Chemical structure of voriconazole (B) postulated chemical structure of voriconazole fragment. Mass spectrum of voriconazole obtained by direct injection of aqueous voriconazole standard using a syringe pump. The MS ion mode is positive. (A) Not fragmented voriconazole: 350.4 *m/z* is the relative mass of voriconazole with a single charge (H^+), 372.3 *m/z* describes the relative mass of voriconazole with a positive sodium ion. A degradation product from voriconazole has the specific mass *m/z* = 281.4. (B) Mass spectrum of the fragmented voriconazole after isolation of the specific mass *m/z* = 350 and fragmentation with an amplitude of 0.8 V. The major voriconazole ion has the specific mass *m/z* = 281.3, which we used for quantification. The other two fragmentation masses (127.3 and 224.3 *m/z*) can be used for additional identification.

of quantitation of 0.2 μg/ml. Column conditioning was carried out using three different solutions: two different solutions for the washing step and methanol-glacial acetic acid (99:1, vol/vol) for elution. The collected eluate was dried and reconstituted in the mobile phase. One chromatography run took 12 min. Detection was done by UV (254 nm). Perea et al. [28] used acetonitrile precipitation followed by reverse-phase HPLC on a C_{18} column. The sample consisted of 0.5 ml plasma and no internal standard. One chromatography run took 10 min. Detection was done by UV (255 nm). The first LC–MS method is described by Zhou et al. [30]. No sample preparation was required because of their clean aqueous humor samples. They detected voriconazole by its mass of 350 *m/z*. Chromatographic separation was conducted on a C_{18} column after 2 μl injection and took 10 min. In a pharmacokinetic study, Walsh et al. [3] refer to a previously validated analytical procedure utilizing automated solid-phase extraction with liquid chromatography for separation of the analytes prior to tandem mass spectrometric detection. However, they cite the paper by Stopher and Gage [29], which makes no mention of MS-detection.

The method given by Stopher and Gage [29] works with three different columns and requires a complex experimental set up. Their simpler method [26], using acetonitrile for protein precipitation, has the disadvantage that it lacks specificity, similar to the method used by Perea et al. [28]. Pennick et al. [27] used complex solid-phase extraction with many different solutions and many manual steps. All these published methods require a large sample volume or clean aqueous samples [30]. The objective of our study was to develop a method that requires only a very small injection volume, is fast and fully automated. Sample pre-treatment should be simple and independent of personnel variation. Additionally, the method has to be specific and reliable for routine therapeutic drug monitoring and laboratory experiments. Such a method should be highly reproducible and suitable for the determination of clinically or laboratory relevant voriconazole levels. For example, the concentration range is c_{min}–c_{max} 0.389–4.695 μg/ml in plasma after a loading dose of 6 mg/kg twice daily [6].

The parallel column-switching technique and online-extraction system presented here is fast and uncomplicated to use. It serves to enhance the sample pre-treatment and extraction step. Pennick et al. [27] needed an SPE conditioning step, washing step, sample evaporation step, etc. However, many pre-treatment steps increase the possibility of interferences and are time consuming. Pennick et al. [27] used an internal standard to reduce these problems. In contrast, we use a fully automated online extraction system.

The LC–MS-integrated sample preparation described here utilizes special column packing materials which allow for direct and repetitive injection of untreated biofluids [31–32]. LiChrospher® RP-ADS belongs to the unique family of restricted access materials (Fig. 2). It has two chemically different surfaces. Hydrophilic, electroneutral diolgroups are bound at the outer surface of the spherical particles with a diameter of 25 μm. This chemically inert layer protects the column from any unwanted contamination caused by interaction with the protein matrix, even if used repetitively. The inner surface of the porous particles is covered with a hydrophobic dispersion phase (C_4, C_8, C_{18} alkyl-chains). These adsorption centres are accessible to low molecular analytes. It has been used successfully for analyses of antibiotics in serum (e.g. [33–34]).

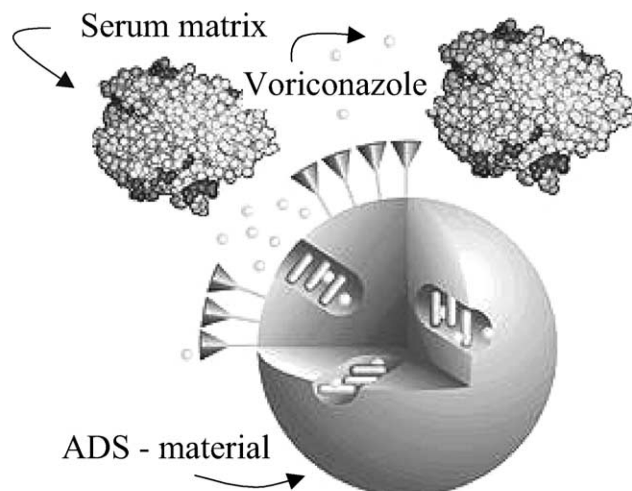

Fig. 2. Scheme of restricted access material (ADS), modified from [36].

2. Experimental

2.1. Chemicals

Voriconazole was obtained from the stock of the Institute of Environmental Medicine and Hospital Epidemiology (Freiburg University Hospital, Germany). Acetonitrile (LiChrosolv®) and formic acid were purchased from Merck (Darmstadt, Germany). Acetonitrile was of gradient grade and formic acid was of analytical grade. HPLC-grade water was generated using a Milli-Q water-purification system from Millipore (Molsheim, France). Pooled blank serum samples were obtained from Freiburg University Hospital. Dulbecco's PBS was obtained from Cell Concepts (Umkirch, Germany).

We prepared a stock standard solution of voriconazole in water at a concentration of 100 μg/ml. Calibration standards at voriconazole concentrations of 0.05, 0.1, 0.5, 1.0, 2.0 and 5.0 μg/ml in pool serum were prepared by appropriate addition of the stock solution to the blank pool serum. The stock standard solution and the blank pool serum were stored at −20 °C until analysis.

2.2. LC–MS-system

Agilent Series 1100 LC (Agilent Technologies, Wallbronn, Germany) equipped with two binary pumps, a degasser, two six-port switching valves and a column oven was used. Samples were injected via an automatic sample injector. The Chemstation software (Agilent Technologies) was used for instrument control.

The MS-system consisted of an Esquire 3000 plus (Bruker Daltonics, Bremen, Germany) with an orthogonal electrospray ionisation source and an ion trap. The software used was Bruker Daltonics esquire 5.1 (Bruker Daltonik GmbH, Bremen, Germany).

2.3. Columns

The in-line-extraction system was constructed with a LiChroCART® cartridge (25 mm × 4 mm) and packed with LiChrospher® ADS C_8, cartridge holder manu-CART®, Merck KG (Darmstadt, Germany). Chromatography was performed on a Nucleodur 100-5 C_{18ec}—125 mm × 4.6 mm, 5 μm analytical column preceded by a guardcolumn CC 8/4 Nucleosil 100-5 C_{18} (both from Macherey & Nagel, Düren, Germany). The column oven was set to 40 °C.

3. Analytical procedure

3.1. Sample preparation

There was no need for manual sample pre-treatment. Serum samples were injected directly into the LC–LC–ESI-MS–MS system. The autosampler was set at an injection volume of 5 μl. Extraction and chromatographic separation of the analyte were carried out automatically by the LC–LC system. The HPLC integrated extraction procedure steps were: (i) sample application and extraction, (ii) transfer of the analyte fraction and chromatographic separation, and (iii) re-equilibration of the extraction column.

The configuration of the LC–LC–ESI-MS–MS-system with the two switching valves is shown schematically in Fig. 3A. The second valve (i.e. valve 1) is only needed for determination of the rate of recovery. The autosampler and pump 1 were used to load 5 μl of serum containing voriconazole onto the extraction column (ADS). The mobile phase of pump 1 was formic acid 0.1% in water (v/v) and was delivered to the ADS-extraction column at a flow-rate of 0.8 ml/min. Voriconazole was retained on the extraction column, while matrix compounds were flushed to waste with the eluent. After 6 min, the matrix had been fully washed out of the extraction column. The software time-schedule automatically switches the high-pressure valve 2 into transfer position (Fig. 3B), thereby coupling the extraction column with the analytical column. The reservoir of pump 2A contained formic acid (0.1%) in water (v/v) and the reservoir of pump 2B contained acetonitrile. The analytical mobile phase was delivered from pump 2A and B in a ratio of 50:50 (v/v). To simplify changing a method we did not use pre-mixed mobile phases. Within 6–10 min, voriconazole was rapidly eluted from the extraction column by back flushing at a flow-rate of 0.4 ml/min. The higher elution power of the acetonitrile desorbed the analyte from the extraction column and transferred the voriconazole desorbed to the analytical column (C_{18}). The second chromatographic separation on the analytical column was accomplished by an isocratic mobile phase gradient (50:50, v/v). 10 min after starting the analysis, the high-pressure valve 2 switched back to the initial position. The extraction column was re-equilibrated by pump 1 with formic acid (0.1%) in water (v/v), while the chromatographic separation on the analytical column is performed

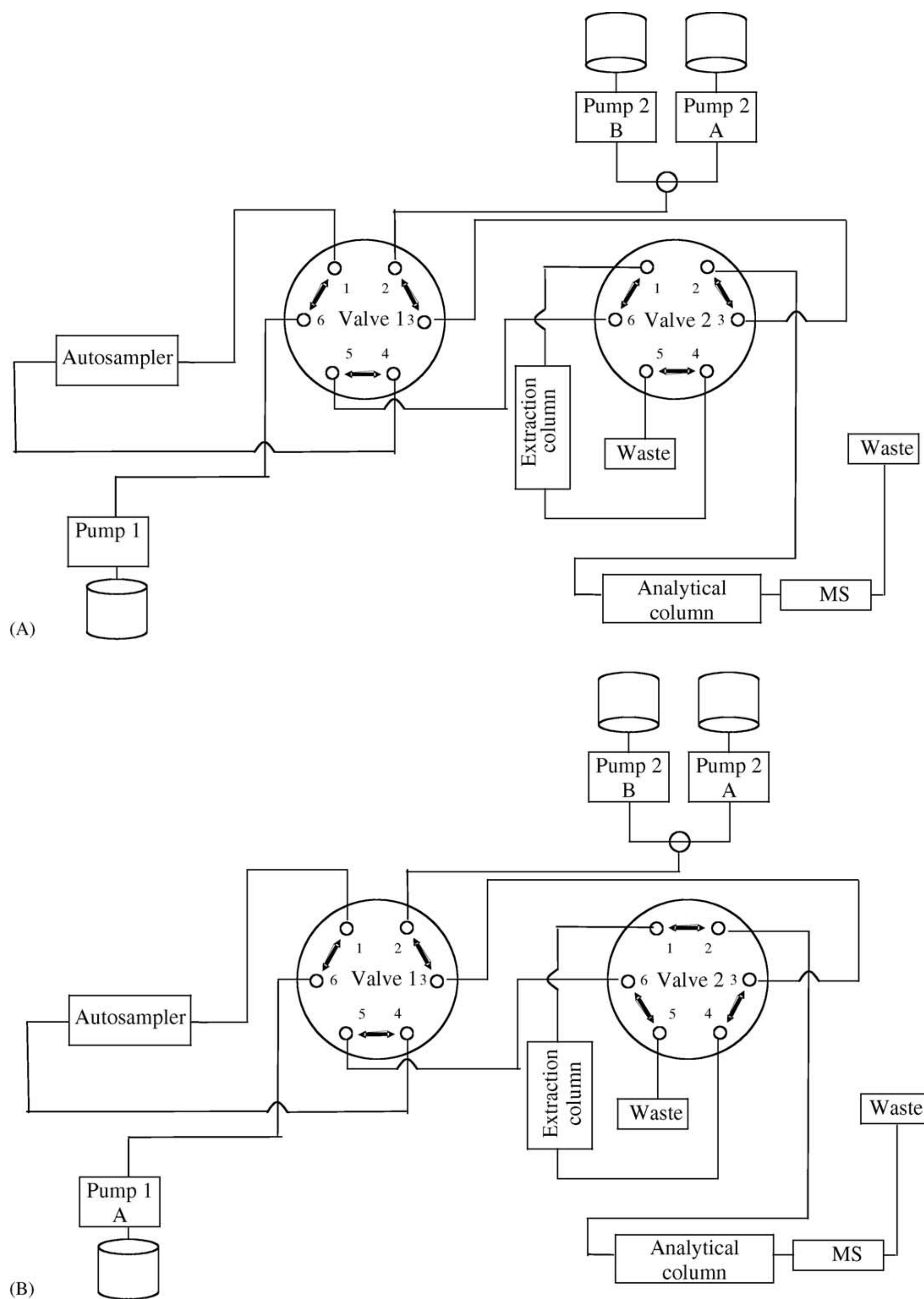

Fig. 3. Scheme of the HPLC-integrated sample preparation. Part (A) shows the system in initial position, ready for sample injection: HPLC–MS circulation is isolated from extraction side. Part (B) displays the transfer and chromatographic separation step: the extraction column is connected with the analytical column.

simultaneously. Total sample preparation and analysis time was 13 min. After 13 min, a new sample was injected and extraction was started, while chromatographic separation of the former sample was finished simultaneously. Parallel extraction and separation is summarized in Fig. 4.

3.2. Detection with mass spectrometry: isolation and fragmentation

The HPLC column effluent was pumped to the ion-trap mass spectrometer equipped with an electrospray ionsource,

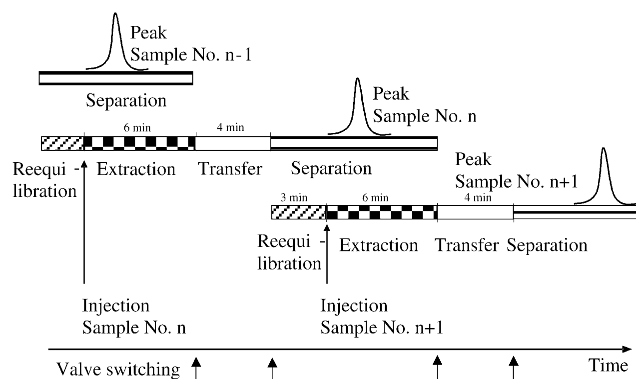

Fig. 4. The timetable of the presented method is schematically shown in this figure. The overlapping analysis sequence is pictured.

which was used in the positive ion mode. The instrument was tuned by direct injection of an aqueous solution of 20 μg/ml voriconazole at 5 μl/min. The following tune parameters were set for optimum voriconazole detection: nebulising gas pressure 50.0 psi (1 psi = 6894.76 Pa); drying gas flow 10.0 l/min; drying temperature 350 °C; spray voltage 4 kV; capillary exit 164.8 V; skimmer 40.0 V; octopole 1 dc 12.0 V; octopole 2 dc 1.70 V; octopole rf amplitude 150.0 V p.-p.; trap drive 43.4; lens 1 voltage −5.0 V; lens 2 voltage −60.0 V. We found the following optimum trap conditions: rolling, on; rolling averages 5 cts; scan begin 100 *m/z*; scan end 1000 *m/z*; maximal accumulation time 200 ms; ion charge control target 150 000; charge control on. Optimum collision energy in the MS–MS mode, corresponding to nearly 100% fragmentation of the protonated molecule, was found to be 0.8 V. Further fragmentation parameters are: isolated specific mass 350.0 *m/z* with width 4.0 *m/z*; smartfrag on; smartfrag start amplitude 30%; smartfrag end amplitude 200%; fragmentation width 10.0 *m/z*, fragmentation time 40 ms; fragmentation delay 0 μs.

3.3. Quantification and validation/qualification

Chromatographic peaks of voriconazole were identified by their retention time and the detected mass of the fragment ion (281.3 *m/z*). The main voriconazole product ion after fragmentation, i.e. MS–MS was 281.3 *m/z*. Other daughter ions were 224.3 *m/z* and 127.3 *m/z*. They showed minor peaks. Quantification was carried out by peak area of the main specific mass peak (281.3 *m/z*).

This LC–LC–ESI-MS–MS assay was validated for linearity of calibration, inter-assay accuracy and precision, quantification limit and specificity of the methodology. The accuracy and precision of the method were assessed by analysing replicates of pool serum samples spiked at different concentrations with within-run and between-run data (see Table 1). The accuracy of the voriconazole serum assay was determined by calculating the mean percentage differences between nominal and measured concentrations. The assay precision was characterised by mean value and coefficient of variation (C.V.) from six replicates of spiked serum samples. Between run precision and accuracy was studied by analysing the peak area of spiked serum samples from six different runs (=inter-run precision). The lower limit of quantification (LLOQ) was defined as being the lowest quantity of analyte determined with a precision and accuracy equal to or better than 20% and an analyte signal at least five times the signal compared to blank (noise) [35]. An analyte signal at least three times the noise of the blank response was defined as the limit of detection (LOD). The calibration curves were based on the peak areas of each standard plotted versus the nominal antimykotic concentration using least squares linear regression. To see possible ion suppression effects of different serum matrices, seven different serum samples were tested. We spiked these matrices with a concentration of 2.0 μg/ml and made a threefold analysis.

4. Results and discussion

4.1. LC–LC

Fig. 3 shows a special column-switching configuration. By using two switching valves we have the opportunity to choose two completely different analytical settings. valve 1 (see Fig. 3) offers two different modes for analysis: one is an analysing mode with column switching, the other without column switching, i.e. an analysing mode like a HPLC system without a switching valve. With voriconazole dissolved in Dulbecco's Phosphate Buffered Saline, we were able to quantify the recovery by using these two different modes for analysis. Injection of the same voriconazole standard using both modes gives a recovery in total of approximately 100%.

By using formic acid (0.1%), we obtained narrow, well-shaped peaks. Therefore, it was possible to extract and detect with the same mobile phase.

Table 1
Statistics of spiked voriconazole serum samples

Nominal concentration (μg/ml)	Within-run				Between-run			
	Precision			Accuracy	Precision			Accuracy
	N	Mean (μg/ml)	C.V. (%)	(%)	*N*	Mean (μg/ml)	C.V. (%)	(%)
0.05	6	0.056	3.65	97.1	6	0.059	16.57	118.6
2.0	6	2.10	6.81	104.7	6	1.88	10.24	94.2
5.0	6	5.39	6.92	109.2	6	5.57	7.10	111.5

C.V.: coefficient of variation; *N*: number of tested samples.

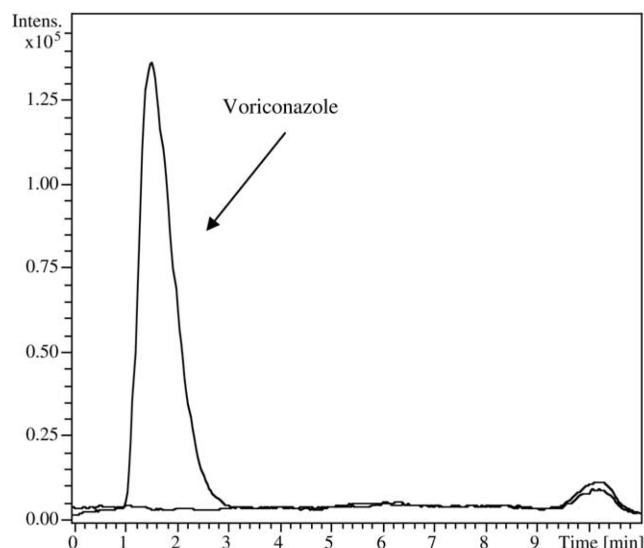

Fig. 5. Chromatograms of spiked matrix vs. blank pooled serum matrix. Serum spiked with 1 μg/ml voriconazole monitored at 281 *m/z*. Both chromatograms are obtained with the described on-line extraction LC–MS method. Voriconazole was isolated and fragmented.

Another important parameter for inline extraction is the transfer time, i.e. the time needed to transfer the substance from the extraction column to the analytical column. A short transfer time is favourable for sharp peaks and fast analysis. Too long a transfer time results in peak broadening. At the same time, all accumulated substances should be completely transferred to the analytical column. We were able to realize a short transfer time of 4 min with a transfer rate of at least 99.8%. A shorter transfer time of 3 min results in a slightly lower transfer rate of 99.5%. Fig. 5 shows a typical chromatogram of pooled serum spiked with voriconazole compared with a blank pooled serum sample.

The analytical LC–LC–ESI-MS–MS method with inline extraction of voriconazole described here eliminates the need for the time consuming and difficult procedures of the method reported previously [27] such as SPE conditioning, washing, sample evaporation, etc. No manual step for sample preparation is required in the method described here. The serum assay is fully automated and allows the determination of voriconazole levels within 13 min. Another advantage of the new method is that only a small quantity of sample is necessary. One analysis only requires 5 μl of serum. All other published methods for the detection of voriconazole from serum use at least 500 μl of serum samples. In some special cases, e.g. pharmacokinetic studies involving children, use of small sample amounts is beneficial to the patients. Currently, it is difficult to obtain a reasonable internal standard. Therefore, another advantage is that no internal standard is required.

4.2. *ESI-MS–MS*

Voriconazole is detected by isolation and fragmentation of the positive charged voriconazole molecule. Its specific mass is $m/z = 350.3$, the specific fragment mass is $m/z = 281.3$ which results from loss of the triazole moiety (Fig. 1B). Voriconazole shows another peak in the mass spectrum (see Fig. 1A). We detected its sodium adduct ($m/z = 372.3$), also.

Roffey et al. showed that voriconazole has different metabolites [13]. Due to the fact of specific mass isolation we could separate the unchanged voriconazole from potential metabolites.

5. Quantification and statistics

Spiked pool serum standards were tested in a concentration range from 0.05 μg/ml to 5.0 μg/ml. The calibration curve was found to be linear over the entire range examined. The equation of the calibration curve was $y = 6661359x + 94397$. x represents the analyte concentration in μg/ml and y the corresponding peak area. The linear regression coefficient was $R^2 = 0.9992$. The precision and accuracy of the voriconazole assay using spiked serum standards is summarized in Table 1. The lower limit of quantification (LLOQ) of the assay is 0.05 μg/ml. The limit of detection (LOD) is 0.03 μg/ml. The linearity range achieved for this assay (0.05 to 5.0 μg/ml) effectively covers the plasma concentrations of voriconazole, which is, for example, c_{min}–c_{max} 0.165–4.309 μg/ml in children (2–12 years old) after multiple dosing of 3 mg/kg [3].

To see possible effects, e.g. on signal intensity of different serum matrices the method was crosschecked with seven different spiked serum samples from seven different patients, not treated with voriconazole. These samples were tested threefold. The C.V. were 4.03%; 9.90% and 3.07%. The C.V. of all seven samples, measured threefold, was 4.09%. Samples of patients treated with voriconazole were tested also. Serum samples from two different patients at seven different time points were analysed. The analysis of the real patient samples showed no differences compared to the spiked serum samples. Therefore, one can conclude the detection with mass isolation and fragmentation results in a high specific targeted detection of unmetabolised voriconazole. Only metabolites with the same chromatographic behaviour and the same mass and formation of the same fragments could interfere. This is highly improbable.

6. Conclusion

The LC–LC–ESI-MS–MS assay presented here allows for the rapid and automated determination of voriconazole in serum without deproteinisation or any other form of pre-treatment. Automated inline-extraction chromatography shows sufficient performance and precision. In addition, the method is very specific. The first step is an intensive automated sample clean up, the second step chromatographic separation and the third step mass sensitive selection and fragmentation followed by detection and quantification of

the specific voriconazole fragment mass. These three steps ensure specific voriconazole detection and effective elimination of potential contaminations. No internal standard and only very small quantities (5 μl) of serum are required. Using a column-switching technique, we were able to analyse the voriconazole contained in the serum without any pretreatment. Combination of the in line extraction technique with column-switching liquid-chromatography (LC–LC) and electrospray ionisation mass spectrometry (ESI-MS–MS) enabled us to detect voriconazole in low concentrations and with maximum specificity. The method fulfils the needs for clinical routine use and for pharmacokinetic studies.

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References

[1] P.H. Chandrasekar, E. Manavathu, Drugs Today 37 (2001) 135.
[2] J.B.G. Stern, Antimicrob. Agents Chemother. 48 (2004) 1065.
[3] T.J. Walsh, M.O. Karlsson, Driscoll, Antimicrob. Agents Chemother. 48 (2004) 2166.
[4] T.J. Walsh, I. Lutsar, T. Driscoll, B. Dupont, M. Roden, P. Ghahramani, M.R. Hodges, A.H. Groll, J.R. Perfect, Pediatr. Infect. Dis. J. 21 (2002) 240.
[5] L. Purkins, N. Wood, D. Kleinermans, K. Greenhalgh, D. Nichols, Br. J. Clin. Pharm. 56 (Suppl. 1) (2003) 17.
[6] L. Purkins, N. Wood, K. Greenhalgh, M.D. Eve, S.D. Oliver, D. Nichols, Br. J. Clin. Pharm. 56 (Suppl. 1) (2003) 2.
[7] I. Lutsar, S. Roffey, P. Troke, Clin. Infect. Dis. 37 (2003) 728.
[8] L. Purkins, N. Wood, P. Ghahramani, K. Greenhalgh, M.J. Allen, D. Kleinermans, Antimicrob. Agents Chemother. 46 (2002) 2546.
[9] H.M. Lazarus, J.L. Blumer, S. Yanovich, H. Schlamm, A. Romero, J. Clin. Pharmacol. 42 (2002) 395.
[10] S.M. Hariprasad, W.F. Mieler, E.R. Holz, H. Gao, J.E. Kim, J. Chi, R.A. Prince, Arach. Ophthalmol.-Chic. 122 (2004) 42.
[11] V. Martinez, J.L. Le Guillou, C. Lamer, M. Le Jouan, M. Tod, F. Dromer, Antimicrob. Agents Chemother. 47 (2003) 3375.
[12] D. Andes, K. Marchillo, T. Stamstad, R. Conklin, Antimicrob. Agents Chemother. 47 (2003) 3165.
[13] S.J. Roffey, S. Cole, P. Comby, D. Gibson, S.G. Jezequel, A.N. Nedderman, D.A. Smith, D.K. Walker, N. Wood, Drug Metab. Dispos. 31 (2003) 731.
[14] M.C. Serrano, A. Valverde-Conde, M.M. Chavez, S. Bernal, R.M. Claro, J. Peman, M. Ramirez, E. Martin-Mazuelos, Diag. Micr. Infect. Dis. 45 (2003) 131.
[15] R.K. Li, M.A. Ciblak, N. Nordoff, L. Pasarell, D.W. Warnock, M.R. McGinnis, Antimicrob. Agents Chemother. 44 (2000) 1734.
[16] F. Marco, M.A. Pfaller, S. Messer, R.N. Jones, Antimicrob. Agents Chemother. 42 (1998) 161.
[17] Pfaller M.A. Messer, S.A. Boyken, Diag. Micr. Infect. Dis. 48 (2004) 201.
[18] M.A. Pfaller, J. Zhang, S.A. Messer, M.E. Brandt, R.A. Hajjeh, C.J. Jessup, M. Tumberland, E.K. Mbidde, M.A. Ghannoum, Antimicrob. Agents Chemother. 43 (1999) 169.
[19] M.A. Pfaller, S.A. Messer, R.J. Hollis, R.N. Jones, D.J. Diekema, Antimicrob. Agents Chemother. 46 (2002) 1723.
[20] International Fungal Surveillance Participant GroupM.A. Pfaller, D.J. Diekema, S.A. Messer, L. Boyken, R.J. Hollis, R.N. Jones, J. Clin. Microbiol. 41 (2003) 78.
[21] M. Ruhnke, A. Schmidt-Westhausen, M. Trautmann, Antimicrob. Agents Chemother. 41 (1997) 575.
[22] S. Perea, G. Gonzalez, A.W. Fothergill, W.R. Kirkpatrick, M.G. Rinaldi, T.F. Patterson, Antimicrob. Agents Chemother. 46 (2002) 3039.
[23] Q.N. Sun, A.W. Fothergill, D.I. McCarthy, M.G. Rinaldi, J.R. Graybill, Antimicrob. Agents Chemother. 46 (2002) 1581.
[24] S. Perea, A.W. Fothergill, D.A. Sutton, M.G. Rinaldi, J. Clin. Microbiol. 39 (2001) 385.
[25] W.R. Kirkpatrick, R.K. McAtee, A.W. Fothergill, M.G. Rinaldi, T.F. Patterson, Antimicrob. Agents Chemother. 44 (2000) 2865.
[26] R. Gage, D.A. Stopher, J. Pharm. Biomed. Anal. 17 (1998) 1449.
[27] G.J.C. Pennick, Antimicrob. Agents Chemother. 47 (2003) 2348.
[28] S. Perea, G.J. Pennick, A. Modak, A.W. Fothergill, D.A. Sutton, D.J. Sheehan, M.G. Rinaldi, Antimicrob. Agents Chemother. 44 (2000) 1209.
[29] D.A. Stopher, R. Gage, J. Chromatogr. B 691 (1997) 441.
[30] L. Zhou, R.D. Glickman, N. Chen, W.E. Sponsel, J.R. Graybill, K.W. Lam, J. Chromatogr. B 776 (2002) 213.
[31] K. Boos, Fresenius J. Anal. Chem. 352 (1995) 684.
[32] R. Trittler, M. Ehrlich, T.J. Galla, R.E. Horch, K. Kummerer, J. Chromatogr. B 775 (2002) 127.
[33] M. Ehrlich, R. Trittler, F.D. Daschner, K. Kummerer, J. Chromatogr. B 755 (2001) 373.
[34] H. Egle, R. Trittler, K. Kummerer, Ther. Drug Monit. 26 (2004) 425.
[35] U.S. Department of Health and Human Services Food and Drug Administration, Bioanalytical method validation, Guidance for Industry, 2001, www.fda.gov/cder/guidance/4252fnl.pdf.
[36] Merck KGaA Darmstadt, LiChrospher® ADS für die HPLC-integrierte Probenaufbereitung, 1995.