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Validation of an LC Method for Therapeutic Drug Monitoring of Voriconazole in Patients

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Abstract

An accurate and precise LC method using diode array detection for the determination of voriconazole in human serum/plasma samples has been developed and validated for use in pharmacokinetic studies.

A harmonized validation strategy based on the accuracy profiles was used as a suitable tool to guaranty the quality of the results obtained by the use of the analytical validated methodology in a routine setting and to ensure the risk of obtaining the future measurements outside the previously fixed acceptance limits.

As pointed recently the FDA, a weighted 1/x² linear regression model ranging from 0.25 to 10.35 mg/L was selected as the simplest calibration model that maximized the accuracy all over the range. Relative bias was < 7%, assay imprecision was always < 4% and mean extraction recovery from plasma was > 90%. So, accuracy did not exceed the acceptance limits settled at ±15% according to the FDA or Washington conference regulatory requirements for bioanalytical methods.

The validated analytical procedure compliants with strongest regulatory standards and their results are rapid and good enough to enable the laboratory to routinely provide useful and accurate pharmacokinetic data in time to adjust patient regimens.

Keywords: HPLC; Therapeutic drug monitoring; Accuracy profile; Voriconazole; Validation

Introduction

Voriconazole (VRC), (2R,3S)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1,2,4-triazol-1-yl)-2-butanol) is a triazole antifungal derived from the structure of fluconazole (Perea et al., 2000). Compared to fluconazole, voriconazole has an enhanced antifungal spectrum with activity against *Aspergillus*, *Candida*, *Scedosporium* and *Fusarium* species (Cuenca-Estrella et al., 1998; Espinel-Ingroff et al., 1998; Chandrasekar et al., 2001; Espinel-Ingroff et al., 2001; Espinel-Ingroff et al., 2007; Clancy et al., 1998; McGinnis et al., 1998; Perfect et al., 2003). Voriconazole, like other azole antifungals, alters the cell membrane function and permeability, resulting in cell dysfunction and growth arrest (Como et al., 1994; Borgers et al., 1980).

Voriconazole undergoes extensive hepatic metabolism, mainly by CYP2C9, CYP2C19 and CYP3A4 isoenzymes (Hyland et al., 2003) and displays non-linear pharmacokinetics due to saturation of first-pass metabolism and systemic clearance (Roffey et al., 2003; Lazarus et al., 2002; Purkins et al., 2002). Elevated voriconazole levels may be caused by hepatic dysfunction, polymorphism of CYP isoenzymes or a number of drugs-inhibitors used concomitantly with this antifungal agent (Boyd et al., 2004; Ikeda et al., 2004; Jeu et al., 2003).

So, although routine monitoring of voriconazole is not indicated, therapeutic drug monitoring (TDM) of patients undergoing therapy with voriconazole may be advisable to guide dosing and to individually optimize the therapy, especially in different physiopathological states, such as in patients with reduced hepatic function or while on concurrent therapy with drugs that affect its metabolism.

Several assays have been reported for the determination of voriconazole in biological fluids including bioassay (Perea et al., 2000), capillary zone electrophoresis (Grego et al., 2001), high performance liquid chromatographic methods (Roffey

et al., 2003; Stopher et al., 1997; Gage and Stopher, 1998; Pascual et al., 2007; P é hourcq et al., 2004; Pennick et al., 2003; Wenk et al., 2006; Khoshsorur et al., 2005; Chhun et al., 2007) one with fluorescencia detection (Michael et al., 2008) or the more sensitive but expensive liquid chromatography mass spectrometry (LC-MS) technologies (Zhou et al., 2002; Keevil et al., 2004; Egle et al., 2005; Vogeser et al., 2005) with a equipment cost not available for most clinical laboratories.

Here, our first objective was to develop a reliable HPLC-UV method for the quantification of voriconazole suitable for therapeutic drug monitoring of patients. Our second objective was to validate the developed methodology in order to check its applicability in the routine area and the future analysis of unknown samples using the validated method. Recently, a novel validation strategy based on the use of accuracy profiles has been introduced (Boulanger et al., 2003; Hubert et al., 2003; Hubert et al., 2004; Hubert et al., 2007a; Hubert et al., 2007b; Hubert et al., 2008). The notion of including the use of accuracy profiles as a decision tool to select the most appropriate response function, to estimate the limit of quantitation or to evaluate the concentration range, is in accordance with the objective of an analytical method that can be summarized as its ability to quantify as accurately as possible each of the unknown quantities in human samples that the laboratory will have to determine.

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So, in the present work, a simple, rapid and accurate HPLC method for determination of voriconazole in plasma/serum has been developed and validated in our laboratory according to the mentioned validation strategy. Moreover, to the best of our knowledge, this is the first account of a voriconazole HPLC assay for which a specific evaluation of the risk of the procedure related to the future use of the method during its daily use for the therapeutic drug monitoring of voriconazole in the clinical setting is reported. Recently, we have published two paper reporting a lamotrigine bioanalysis methods validated according with this strategy (Zufía et al., 2009).

Material and Methods

Chemical and reagents

Voriconazole reference standard was kindly supplied by Pfizer Central Research (Sandwich, UK). Ketoconazole (98% minimum purity) used as internal was purchased from Sigma-Aldrich (Steinheim, Germany).

99.9% purity HPLC grade solvents methanol and acetonitrile were obtained from Merck (Barcelona, Spain). Potassium dihydrogen phosphate and triethylamine, analytical grade, were purchased from Panreac (Barcelona, Spain).

For method validation, human plasma (University hospital blood bank) to prepare calibration, validation and quality controls standards and to study the specificity of the method was obtained from pooled drug-free samples collected from healthy volunteers.

Drug solutions

Stock standard solutions of voriconazole and the internal standard were prepared by dissolving appropriate amounts of compounds in a known volume of methanol and stored at -30°C.

Working standard solutions for the calibration, validation and quality controls standards were prepared by appropriate dilutions of two independent stock standard solutions in blank human plasma obtained from the University hospital blood bank.

Sample preparation by solid-phase extraction procedure (SPE)

Blood patient samples were collected in tubes DB Vacutainer SST systems and transported to the pharmacokinetic laboratory where they were centrifuged at 1500g for five minutes for serum separation. The serum was immediately processed for the solid-phase extraction procedure described below.

Different anticoagulants have been tested with no evidence of any difference in Voriconazole determination.

The procedure of extraction of the analyte from plasma/serum was a solid-phase extraction pretreatment developed in our laboratory.

Briefly, a volume of 500 µL of a unknown plasma/serum sample, calibration, validation or quality control sample was placed in a 1.5 mL centrifuge tube and 100 µL of a plasma solution of ketoconazole (50 mg/L) as internal standard and 200 µL of water were added and homogenized by vortex-mixed.

Waters OASIS HLB cartridges (30 mg, 1 mL) were used for voriconazole sample pretreatment. The solid-phase extraction procedure (SPE) was carried out in a Tecknokroma Vac Elut apparatus according to the following steps: a) conditioning with 1 mL methanol; b) equilibration with 1 mL water; c) loading of 900 µL of the mixed plasma/serum sample; d) washing with 1 mL

water; e) elution with 1 mL acetonitrile/methanol (90/10, v/v) mixture. The eluate was then dried under vacuum at 50°C in a vortex evaporator. The residues were reconstituted in 100 µL of mobile phase and after vortex-mixing for 20 s, these samples were centrifuged at 10,000 g for 3 min at room temperature. The clear supernatant was transferred to microvials and the autosampler programmed to inject 50 µL into the HPLC system.

High-performance liquid chromatography

The chromatographic system used in the study was a Hewlett-Packard 1100 series with a Model G1311A quaternary pump, Model G1313A autosampler, Model G1315A diode array detector, Model G1316A column compartment and Model G1322A degasser. Data were acquired and processed with HP Chem Station chromatography manager software from Agilent Technologies (Santa Clara, CA, USA).

Separation of compounds was achieved using a Atlantis® dC 18 (3 µm, 4.6 mm i.d. x 75 mm) analytical column protected by a Atlantis® dC 18 precolumn.

The chromatographic separation was carried out using a mobile phase consisting of a mixture of 1mM potassium dihydrogen phosphate with 0.6 % TEA and an acetonitrile gradient from 30 to 70% in five minutes at a constant flow rate of 1.2 mL/min. The total run time was 8 min.

The column was maintained at 40°C and the eluent was monitored at a wavelength of 254 nm.

Calibration and validation standards

In order to validate the analytical method, we prepared two kinds of samples for calibration and validation in an independent way.

The calibration standards consist of plasma/serum samples, containing known concentrations of the analyte of interest. The samples are only used for calibration and they were prepared according to the protocol that will be applied routinely. Two calibration standards series of eight concentrations levels replicated on three different days were performed. Spiked plasma samples used as calibration standards (0.26, 0.52, 0.78, 1.04, 2.6, 5.2, 7.8, and 10.35 mg/L) were prepared by addition of different volumes of the corresponding standard solution of voriconazole in blank human plasma obtained from the University hospital blood bank. The most appropriate response function was selected according to the accuracy profile approach in order to guaranty a reliable quantification.

The validation standards are also matrix samples containing known concentrations of the analyte of interest. They were independently prepared in the matrix simulating as much as possible the future routine analysis of voriconazole samples. In the validation phase, the validation standards represent the future samples that the analytical procedure will have to quantify. The concentration levels selected for the validation standards were 0.26, 0.78, 2.6, 7.8 and 10.35. Seven replicates were prepared at each concentration level for two days.

Quality control samples were prepared in human plasma at the concentrations of 0.78, 2.6 and 7.8 mg/L, as described above for the calibration and validation standards. Calibration and validation standards and quality control samples were analyzed in the same way as patient plasma samples.

Application to clinical pharmacokinetic studies

To demonstrate that this HPLC method is applicable to pharmacokinetic studies, it has been used to determine

voriconazole concentrations in plasma/serum from patients receiving voriconazole endovenous or orally in the Clínica Universidad de Navarra (Spain).

Results and Discussion

HPLC assay development

Several HPLC methods have been reported for the determination of voriconazole concentrations in human plasma/serum. These use generally reversed-phase procedures after plasma protein precipitation with acetonitrile (Perea et al., 2000; Gage and Stopher, 1998; Pacual et al., 2007) or liquid-liquid extraction (Khoschorur et al., 2005; Langman et al., 2007; Michael et al., 2008) as sample preparation previous to reversed chromatography. Only the method of Gage and Stopher, (1998) was based on size exclusion chromatography coupled with on-line reverse phase chromatography in a complex and difficult method that involves column-switching of three columns. To our knowledge, also there is only one published method that utilizes solid phase extraction (Pennick et al., 2003) with C18 silica-based cartridges for sample pretreatment. Gordien et al. (2009) have recently published a solid phase extraction procedure automated in a sample processor using polymeric cartridges to a simultaneous determination of azoles. Deproteinization by precipitation is a simple and rapid sample preparation methodology but the possibility of eventual loss of analytes by drug entrapment in the precipitant may decrease considerably recovery. Liquid-liquid extraction is tricky, tedious and time consuming and also use a considerably high amount of organic solvents. Additionally, Pélouchery et al. (2004) or Zhou et al. (2007) used direct injection of untreated samples.

Here, we describe a rapid and robust assay with solid phase extraction based on polymeric packings to determine voriconazole. Different extraction procedures for voriconazole and the internal standard were tested in our laboratory (data not shown) and the best results were found using Oasis HLB (hydrophilic-lipophilic balanced copolymer) cartridges. As a polymeric, it is free from the complicating interactions with silanols and due to its hydrophilic properties, it has no impact of sorbent drying showing less assay variability and tighter RSD's than with silica-based reversed-phase packings. The procedure was done according to the sample preparation treatment described in Material and Methods section. The extraction yield data obtained with the Oasis HLB cartridges were very high and reproducible (Table 1). Our results were similar to the published by Michael et al. (2008) using a liquid-liquid extraction, but with our SPE procedure, the reduction in organic solvent usage is a more environmentally friendly practice.

During the phase of optimization of the assay, different stationary and mobile phase compositions were evaluated for their performance in separating voriconazole, its internal standard, ketoconazole, and endogenous compounds. The mixture already described above of a buffer 1 mM potassium dihydrogen phosphate containing 0.6 % triethylamine and a gradient of five minutes of acetonitrile as organic modifier

using a Atlantis® dC 18 (3 µm, 4.6 mm i.d. x 75 mm) produced optimal separation with retention times of 4.0 and 5.1 min for voriconazole and ketoconazole, respectively. The use of a shorter column (75 mm) packed with smaller particles (3 µm) suppose a substantial solvent and time savings. Under these conditions both compounds exhibit good retention and very sharp and symmetrical peak shapes. Typical chromatograms obtained with extracted drug-free human plasma, samples of plasma spiked with voriconazole (0.25 and 5 mg/L) and internal standard (40 mg/L) and one patient's plasma treated with a maintenance dose of endovenous voriconazole of 240 mg twice daily and spiked with internal standard (50 mg/L) are illustrated in Figure 1.

Assay validation

In the present study, the recent approach using accuracy profiles is applied (Boulanger et al., 2003; Hubert et al., 2003; Hubert et al., 2004; Hubert et al., 2007a; Hubert et al., 2007b; Hubert et al., 2008; Gonzalez and Herrador, 2006). It is based on β -expectation tolerance intervals for the total error measurement that includes trueness (bias) and intermediate precision (standard deviation). The first advantage of this strategy is the possibility to guarantee that the results of the measurement that will be obtained during the future use of the validated method will be included within the acceptance limits previously fixed. So, this strategy helps us to select the most appropriate method to be used in routine for therapeutic drug monitoring. In our work, β was 95 %, indeed, it guarantees that at least, the 95 % of future results will be included in the ± 15 % settled according to the FDA or Washington conference regulatory requirements (Guidance for industry: Bioanalytical Method Validation, May 2001; Shah et al., 1992a,b; Shah et al., 2000). So, this strategy helps us to determine the acceptability of a method to be used in routine for therapeutic drug monitoring.

According to this strategy, the analytical methodology was validated in order to ensure the reliability of the developed method in terms of selectivity, response function, absolute recovery, linearity, limits of quantification and detection, trueness, precision and accuracy. The validation data were processed by e-nova® software Version 2.0 (Arlenda, Liège, Belgium).

Selectivity

A useful analytical method should permit resolution and detection of the analytes of interest and the internal standard from other interfering metabolites and co-eluting endogenous compounds or co-administered therapeutic drugs. So, standard solutions of several antibiotics or other drug agents which could be co-administered in polytherapy during the voriconazole treatment of patients, were injected confirming the good method selectivity because no interference was found with all tested drugs that could be co-administered. Also, possible interferences of other antimycotics were investigated by analyzing pure solutions in mobile phase of fluconazole or itraconazole which caused no interferences with voriconazole or the internal standard selected, ketoconazole.

Possible interferences from endogenous constituents of human plasma were evaluated by analyzing a minimum of six pooled blank plasma samples without internal standard obtained from different healthy volunteers. The degree of interference was assessed via diode-array to verify that each observed peak eluted free of any potential interference. No interfering peaks were observed and no significant peaks were found at the retention times of the analyte or the internal standard. Interferences that appear at the top of the chromatogram

Concentration (mg/L) (n=7)	Recovery (%)
0.25	104.6
0.78	103.4
2.60	101.3
7.76	105.5
10.35	102.2

n= replicates

Table 1: Absolute recoveries for voriconazole extraction with OASIS HLB cartridges.

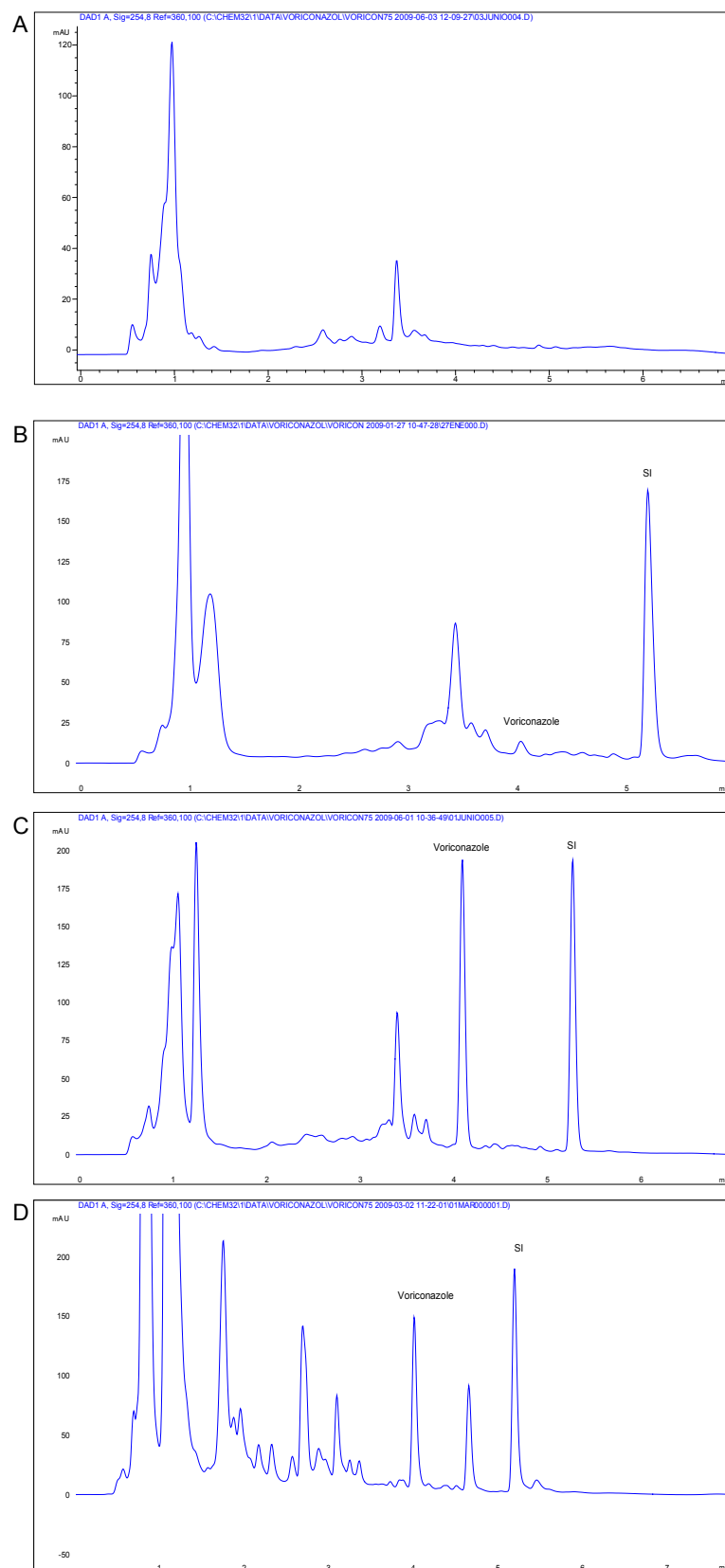


Figure 1: Chromatographic separation of a blank plasma extract A) without voriconazole, from a blank plasma extract B) at the LLOQ (0.25 µg/ml) and C) after the addition of a concentration of 5 µg/ml of voriconazole and 50 µg/ml of its internal standard and D) from a patient plasma extract in treatment with 200 mg twice daily of voriconazole.

probably due to endogenous constituents of human plasma, apparently did not drop too much column lifetime because we have reached more than 200 samples analyzed without a significant loss in efficacy.

Figure 1 shows a representative chromatogram from a

human plasma extract without voriconazole, from a plasma extract spiked with internal standard and with voriconazole at the quantification limit (0.25 mg/L) and at a concentration of 5 mg/L and from a human plasma extract of a patient in treatment with 240 mg twice daily of endovenous voriconazole.

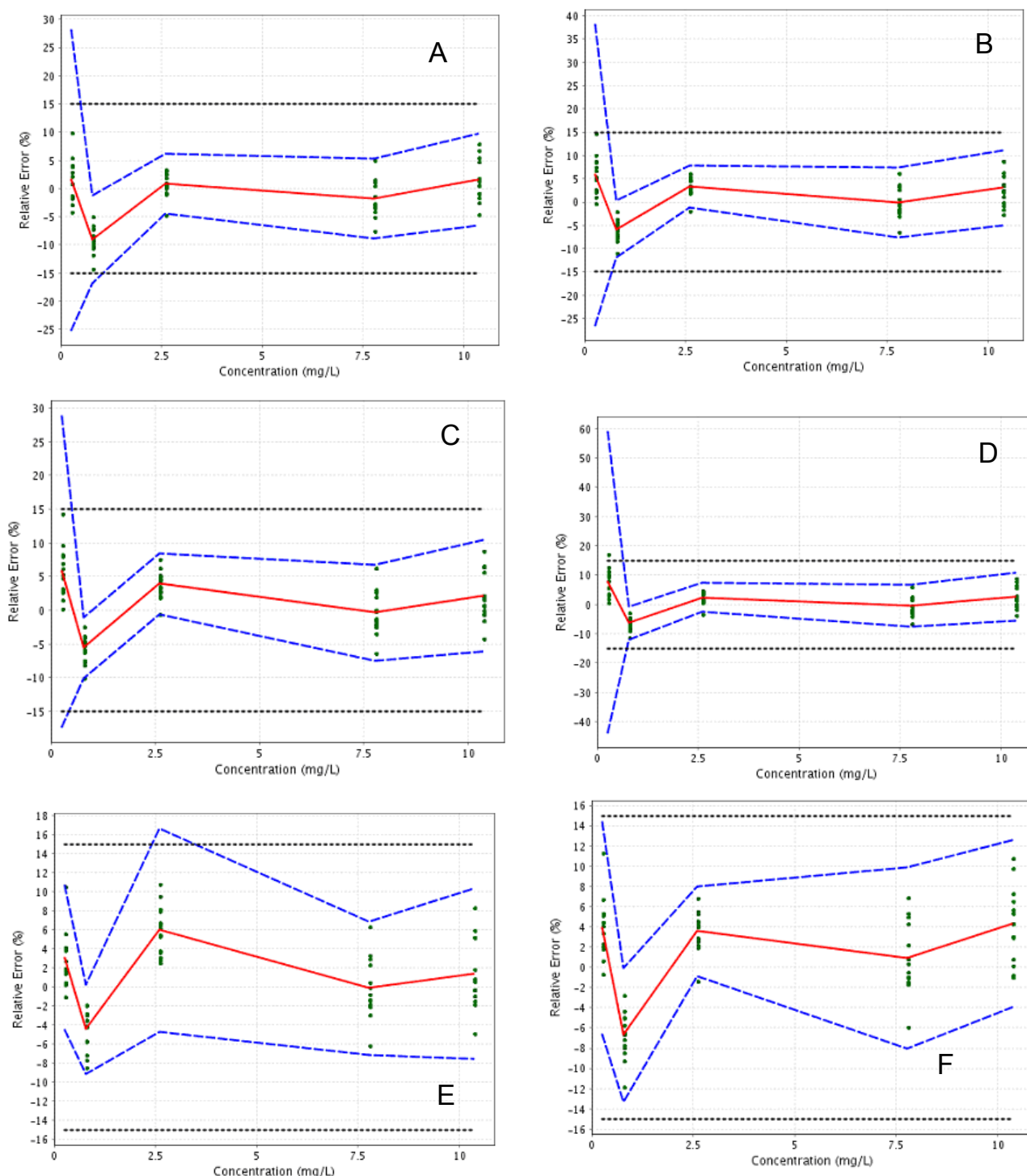


Figure 2: Accuracy profiles for voriconazole using A) a simple linear regression through 0 model, B) a linear regression after logarithm transformation model, C) a weighted $1/x$ quadratic regression model, D) a weighted $1/x$ linear regression model, E) a weighted $1/x^2$ quadratic regression model or F) a weighted $1/x^2$ linear regression model. The plain line is the relative bias, the dashed lines are the β -expectation tolerance limits and the dotted curves represent the acceptance limits (15%). The dots represent the relative back-calculated concentrations and are plotted with respect to their targeted concentration.

The approximate retention times for voriconazole, and the internal standard were 4.0, and 5.1, respectively.

Response function

This step is decisive since the reliability of the future validation results will depend on the selected regression model. The response function was evaluated from three calibration curves corresponding to three days, constructed from two calibration standards for each concentration level, eight, ranging from 0.26 to 10.35 mg/L. Several regression models were fitted in order to analyze the relationship between concentration and analytical response. Independent validation standards, five, at 0.26, 0.78, 2.6, 7.8 and 10.35 mg/L (respectively the lower limit of quantification (LOQ), low, medium and high quality controls and the higher limit of quantification (HLQ)) were also processed. The concentration of the validation standards were back-calculated for each response function in order to determine, by concentration level, the mean relative bias and the upper and the lower β -expectation tolerance limits by considering the estimation of the standard deviation for intermediate precision. From these data, different accuracy profiles were plotted to select the most suitable regression model (Figure 2). As we have mentioned, the acceptance limits were settled to $\pm 15\%$, since we are with a bioanalytical method and considering a risk of 5%. Linear regression through 0, weighted $1/x$ quadratic or linear regression, weighted $1/x^2$ quadratic regression or linear regression after logarithm transformation models were rejected because the lower β -expectation tolerance limit was out of the acceptance at the lowest concentration level. As we can see in Figure 2, there is only one regression model, the weighted $1/x^2$ linear regression model, that met expectations, the β -expectation tolerance intervals was comprised within the acceptance limits all over the concentration range. So, we selected this model to evaluate the different validation criteria because of the objective of the method, the ability to quantify voriconazole in the 0.26-10.35 mg/L range remained fulfilled.

Absolute recovery

Absolute recoveries (%) were determined according to the ratio of the areas of extracted samples treated according to the described procedure to those found after the direct injection of corresponding mobile phase standard solutions containing voriconazole at the same concentrations. We extracted six samples of each concentration and injected them on the same day. Absolute recovery was also evaluated for the internal standard, ketoconazole, from of the extraction of ten samples at a concentration of 50.0 mg/L. Recoveries of voriconazole and

its internal standard were 101.2 %-105.5 % (n=6), and 91.3 % (n=10) respectively. Recoveries for voriconazole were not only high, but also similar for every concentration studied all over the range (Table 1).

Trueness

Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one (Hubert et al., 2003; ISO5725-1, Geneva, Switzerland). As can be seen in Table1, trueness is expressed in terms of absolute or relative bias (%) and was assessed by means of seven validation standards at five concentration levels ranging from 0.25 to 10.35 mg/L during two days. According to regulatory requirements (Guidance for industry, May 2001), trueness was good since the bias ($\leq 7\%$) did not exceed the value of $\pm 15\%$, irrespective of the concentration level.

Precision

The precision of the method is evaluated by calculating the relative standard deviation for repeatability (RSD %) and intermediate precision (RSD %) at each concentration level of the validation standards. The RSD values presented in Table 2 were low for both the repeatability and the intermediate precision ($< 4\%$), demonstrating the good precision of the developed method in accordance with the FDA requirements.

Accuracy and the lower limit of quantification and detection

Accuracy refers to the closeness of agreement between the test result and the accepted reference value or the conventionally true value. Takes into count the total error, i.e. systematic and random errors, related to the test result (Hubert et al., 2003; ISO5725-1, Geneva, Switzerland; Shah et al., 1992a,b). It is represented from the accuracy profile illustrated in Figure 2E. As can be seen from the results in Table 2, the different limits of tolerance of bias (the upper and lower β -expectation tolerance limits) did not exceed the acceptance limits settled at $\pm 15\%$ for each concentration level. Consequently, the proposed method was accurate over the concentration range investigated.

Usually, only the lower limit of quantitation (LLOQ) is defined as the smallest quantity in the sample that can be assayed under experimental conditions with a well defined accuracy (Boulanger et al., 2003; Hubert et al., 2003; Hubert et al., 2004; Hubert et al., 2007a; Hubert et al., 2007b; Hubert et al., 2008). The concept of total error also introduces the

Trueness (n=7, p=2)	Absolute bias (mg/L)	Relative bias (%)
0.25	0.01001	3.868
0.78	-0.05193	-6.690
2.60	0.09215	3.561
7.765	0.07205	0.9282
10.35	0.4470	4.319
Precision (n=7, p=2)	Repeatability (RSD%)	Intermediate precision (RSD%)
0.25	2.499	3.277
0.78	2.060	2.432
2.60	1.984	1.984
7.765	3.322	3.644
10.35	3.679	3.679
Accuracy (n=7, p=2)	Beta-expectation tolerance limit (mg/L)	Relative Beta-expectation tolerance limit (%)
0.25	[0.2415,0.2960]	[-6.673,14.41]
0.78	[0.6729,0.7757]	[-13.31,-0.06648]
2.60	[2.564,2.795]	[-0.9023,8.025]
7.765	[7.137,8.532]	[-8.061,9.918]
10.35	[9.940,11.65]	[-3.961,12.60]

n= replicates; p=days

Table 2: Validation results referred to voriconazole by considering weighted $1/x^2$ linear regression model.

upper limit of quantification (ULOQ) given by the intersection between the accuracy profile and the upper acceptance limit. The intersection with the lower limit defines the LLOQ. In our developed method, according to these results, as the accuracy profile was included inside the acceptance limits (Figure 2C), the lowest concentration investigated (0.25 mg/L) was considered as the LLOQ and the highest concentration investigated (10.35 mg/L) was the ULOQ. Compared with the published HPLC UV (Roffey et al., 2003; Stopher et al., 1997; Gage and Stopher 1998; Pascual et al., 2007; P é hourcq et al., 2004; Pennick et al., 2003; Wenk et al., 2006; Khoschsorur et al., 2005; Chhun et al., 2007) or even fluorescence (Michael et al., 2008) methods, this limit of quantification is similar. Although the fluorescence method of Michael et al. (2008) uses a sample volume of 300 μ L. However, the LC-MS or LC-MS/MS methods described (Zhou et al., 2002; Keevil et al., 2004; Egle et al., 2005; Vogeser et al., 2005) are highly sensitive but too expensive and with a cost equipment not available to most laboratories. Moreover, the linear range obtained effectively covers what is currently believed to be the clinically relevant range for voriconazole concentrations in plasma/serum samples of patients.

The limit of detection (LOD) is the smallest quantity that can be detected, but not accurately quantified in the sample (Boulanger et al., 2003; Hubert et al., 2003; Hubert et al., 2004; Hubert et al., 2007a; Hubert et al., 2007b; Hubert et al., 2008). The LOD (0.078 mg/L) was estimated using the mean intercept of the calibration model and residual variance of the regression.

Linearity

The linearity of an analytical method is its ability within a given range to obtain results directly proportional to the concentrations of the analyte in the sample (Boulanger et al., 2003; Hubert et al., 2003; Hubert et al., 2004; Hubert et al., 2007a; Hubert et al., 2007b; Hubert et al., 2008). For all three series, a regression line was fitted on the calculated concentrations versus the introduced concentrations by applying the linear regression model for which the determination coefficient (r^2), the slope and the intercept are 0.997, 1.036 and -0.044 respectively. The absolute β -expectation tolerance limits were within the absolute acceptance limits demonstrating the linearity of the present method.

Stability

Standards solutions in methanol and quality controls in plasma were evaluated for analyte stability at room temperature, frozen at -30°C and after freeze-thaw cycles. Comparing to freshly made standards, samples showed less than 15% deviation from the initial concentration, indicating stability at room temperature for up to 24 hours. Samples were able to undergo three freeze-thaw cycles and were stable at -30°C for 6 months. Stability of voriconazole beyond this was not evaluated.

Sample extract stability was evaluated by assaying samples on day 0 and re-injecting the samples after 24 hours at room temperature on the autosampler. The samples showed no perceptible degradation.

Dilution and memory effect

No memory effect was detected since voriconazole was not found in blank samples injected after the highest calibrator.

There were no differences in the determination of a sample with a theoretical concentration of Voriconazole of 20 mg/L after a dilution 1/2 or 1/3. Accuracy ranged from 90 % to 110 % and precision was always less than 5 %.

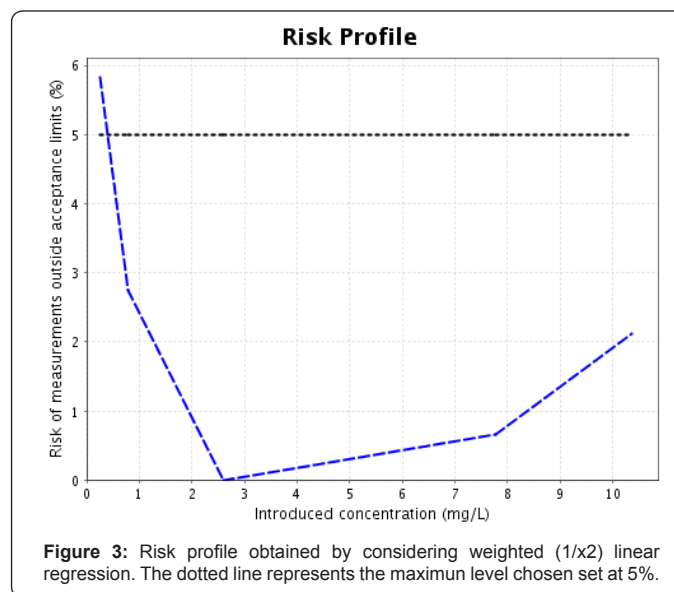


Figure 3: Risk profile obtained by considering weighted (1/x²) linear regression. The dotted line represents the maximum level chosen set at 5%.

Application of the analytical method to biological samples in clinical pharmacokinetic studies

We developed our method to determine voriconazole concentrations in plasma/serum of patients in treatment with this drug orally or endovenously in the Clínica Universidad de Navarra (Spain). This assay was useful to quantify plasma/serum levels of voriconazole in patients undergoing therapy with this antifungal. None of the determined samples posed any matrix interference and no problem for the analyte quantification was found. The chromatogram obtained by injecting a plasma sample from a patient under voriconazole therapy is shown in Figure 1D. This patient (female) took 240 mg of endovenous voriconazole twice daily. A serum sample was collected at 8.00 h in the morning just before the first daily drug administration. We have confirmed the applicability of our method which provides fast quantitative results with specificity, accuracy, and precision. Internal quality control has been assessed for over one year during which time we measured over 20 patients. All controls were essentially found to provide voriconazole concentrations within the target range according to the FDA or Washington conference regulatory requirements (Guidance for industry, May 2001). An accurate and precise quantification of drugs and a fully validated analytical method to determine the drugs is critical in therapeutic drug monitoring. Detailed pharmacokinetic data will be reported in a separated article.

Profile of risk

The risk profile expresses the expected probability to have measurements falling outside the acceptance limits during routine use and is computed according to Mee (1998).

The risk was investigated at each concentration level of the validation standards by taking into account the most appropriate regression model previously determined by use of the accuracy profile as decision tool. The maximum risk tolerated was set at 5 %. Figure 3 shows the risk profile.

Conclusions

We report a accurate assay for the simple and rapid determination of voriconazole. The assay was successfully validated using the approach based on the accuracy profile, strategy proposed by the SFSTP commission which was in

compliance with regulatory documents, including the FDA guidance (Guidance for industry, May 2001). So, this approach has been applied to demonstrate the ability of our developed method to quantify voriconazole.

The validated method has a sufficiently rapid turnaround time and their results are good enough to enable the laboratory to routinely provide useful and accurate pharmacokinetic data in time to adjust patient regimens. Moreover, the profiles of risks were also investigated in order to evaluate the probability that a future measurement obtained during routine use of the method will fall outside the defined acceptance limits. This was a very interesting tool to assist the method development and the quality assurance because it allows us to assess the reliability of our analytical method according to its intended use. Apart from therapeutic drug monitoring, the assay should facilitate pharmacokinetic research in clinical laboratory settings with a low consumption of organic solvent. Clinical pharmacokinetics of voriconazole will be studied in detail in further investigations.

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