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

Hannes Egle, Rainer Trittler, Armin König, Klaus Kümmerer*

Institute of Environmental Medicine and Hospital Epidemiology, Freiburg University Hospital, Hugstetter Str. 55, D 79106 Freiburg i. Br., Germany

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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.

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

* Corresponding author. Tel.: +49 761 270 5464; fax: +49 761 270 5485.
E-mail address: klaus.kuemmerer@uniklinik-freiburg.de (K. Kümmerer).

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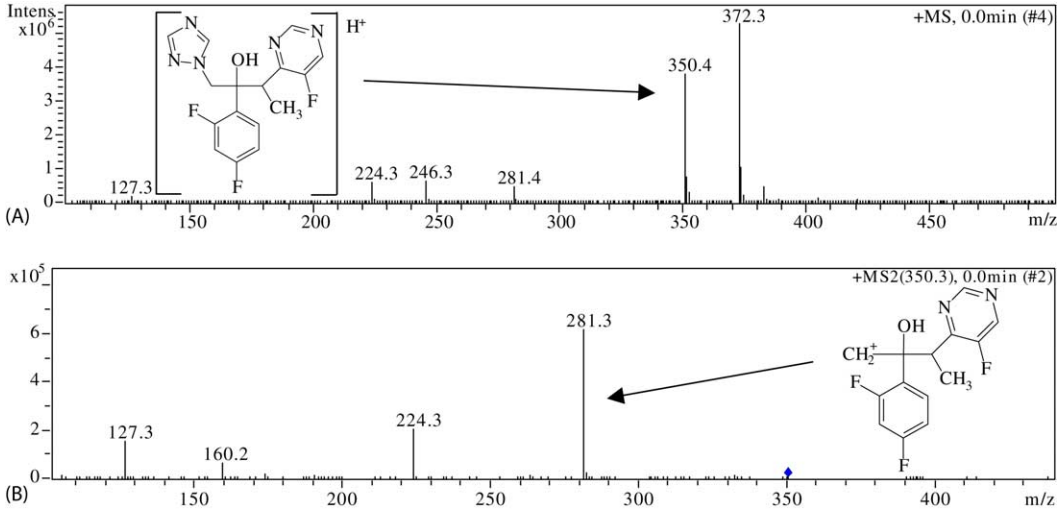

Fig. 1. (A) Chemical structure of voriconazole (B) postulated chemical structure of voriconazole fragment. Mass spectrum of voriconazole obtained by direct injection of aqueous voriconazole standard using a syringe pump. The MS ion mode is positive. (A) Not fragmented voriconazole: 350.4 *m/z* is the 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]).