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Column-switching chromatographic determination of itraconazole and its metabolite hydroxy-itraconazole in human serum

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A column-switching HPLC assay is described for the determination of the antifungal agent itraconazole and its main metabolite hydroxy-itraconazole in serum samples. Three precolumns packed with alkyl-diol silica sorbents differing in alkyl chain length are compared in the sample clean-up step. Chromatographic separation is achieved with a Symmetry C8 column. The assay presented shows a robust and selective analytical procedure with low requirements for sample quantity, no manual sample treatment and high sample throughput.

1. Introduction

Itraconazole (IC) (±)-1-[(*R**)-sec-butyl]-4-[*p*-[4-[*p*-[[(2*S**, 4*R**)-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]-phenyl]-2-1,2,4-triazolin-5-one is a triazole antifungal agent demonstrating a broad spectrum of activity against most human fungal pathogens. It has been shown to be effective in a wide range of superficial mycoses as well as in systemic deep fungal infections [1, 2]. It metabolises by side chain hydroxylation to hydroxy-itraconazole (HIC) appearing in concentrations nearly twice of that of the parent compound. This hydroxy-metabolite contributes importantly to the *in vivo* and *in vitro* activity of itraconazole [3] which explains discrepancies between chromatographic and bioassay methods [2, 4].

Therapeutic drug monitoring of IC and HIC is necessary due to the significant variation in serum concentrations in a variety of population [5], and due to the correlation between drug concentrations and therapeutic efficacy [1]. Several bioanalytical and HPLC assays have been proposed for IC and HIC determination in serum, plasma, and tissue samples. The bioassays proposed lack sensitivity or are unable to distinguish metabolites from the parent compound [6, 7].

Earlier HPLC assays allowed the determination of itraconazole only [6, 8–12], but papers published in the last six years have described HPLC assays which are able to determine both compounds of interest – IC and HIC [1, 2, 13–18]. Sample preparations for most of the HPLC methods are based on multi-step liquid-liquid extraction [9, 10, 14, 17], solid-phase extraction [12, 18] or protein precipitation [1, 13, 15, 16]. All preseparation procedures require tedious manual work with hazardous biological materials and call for least 500 µl of serum sample. There is only one procedure allowing the determination of IC and HIC in serum volumes less than 150 µl [13].

In this paper, a column-switching assay is described using restricted access materials (RAM) for the determination of IC and HIC allowing repetitive direct injection of serum samples without previous manual sample pretreatment.

Alkyl-diol silica (ADS) stationary phases with various hydrophobic internal surfaces were used as precolumns in this work. They belong to the group of RAM allowing the direct injection of untreated biological fluids and having the external hydrophilic surface covered with glyceryl-propyl (diol) phase and the internal hydrophobic one with alkyl chains [19]. The ADS packings differ in the length of the alkyl chain – ADS RP-18, RP-8, and RP-4. The hydrophobicity of the internal surface could affect the selectivity and the efficiency of the preseparation step. These precolumn materials have been used in various applications mainly in the column-switching mode [20–26], or incorporated as a part of a post-column derivatization reactor [27].

2. Investigations, results and discussion

The aim of this study was to develop a chromatographic assay for the determination of itraconazole and its metabolite with direct injection of the serum sample. A column switching system in back-flush mode using restricted access material precolumns was chosen (Fig. 1). The method was developed by the following steps: 1. The mobile phase for washing was chosen to remove all proteins and endogenous compounds from the sample matrix; 2. The precolumn packing material was selected in order to trap the analytes of interest and to enhance the selectivity of the sample clean-up step; 3. In the selection of the analytical column, a peak asymmetry value and the mobile phase requirements were emphasised.

The following mobile phases for washing were tested in a column-switching mode:

- water;
- 25 mM and 50 mM phosphate buffer (pH 7.2);
- 10% methanol, 10% acetonitrile, 10% 1-propanol and 10% 2-propanol in water;
- 10% 2-propanol in 25 mM and 50 mM phosphate buffer (pH 7.2).

The best chromatographic separation (no interferences) was achieved using 50 mM phosphate buffer (pH 7.2).

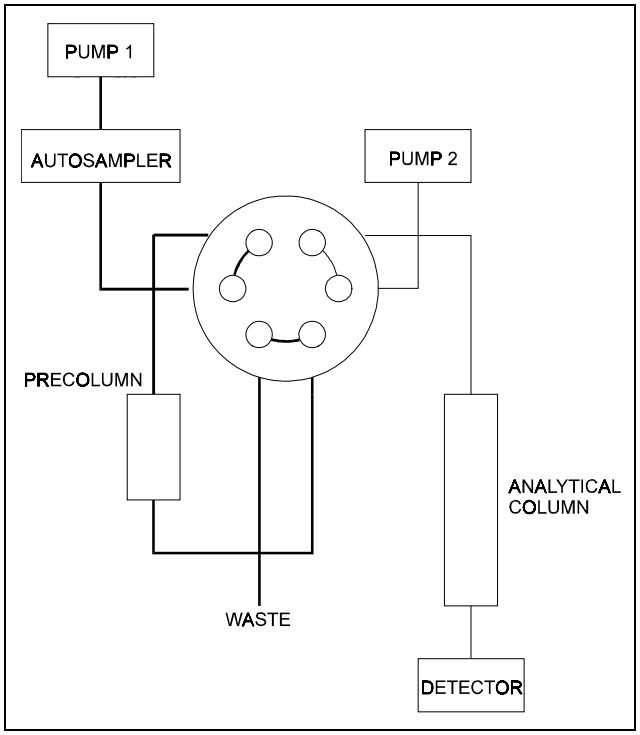

Fig. 1: Instrumental set-up in the LOAD position

The addition of 2-propanol did not improve the selectivity of the preseparation procedure. As reported in our previous paper [26], a volume of 10 ml of washing mobile phase removed all proteins from the serum samples.

Different alkyl chain lengths of the hydrophobic interior influence the preconcentration capability and selectivity of the sample clean-up step.

Preconcentration capabilities of all the precolumns investigated were tested in single column mode, when the analytes were injected directly into the precolumn. The dependence of the capacity ratio of IC on mobile phase composition (from 40% to 90% of 2-propanol in water) is shown in Fig. 2. The retention of IC is very high for the mobile phases I containing less than 40% of 2-propanol

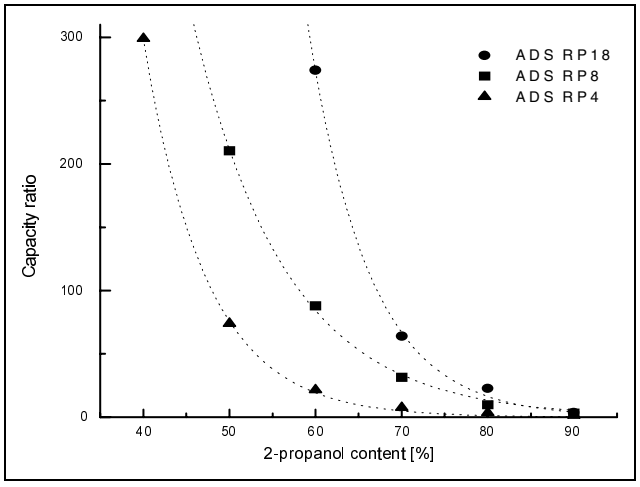

Fig. 2: Dependence of capacity ratio on 2-propanol content
Columns: LiChrospher ADS RP-18, RP-8, RP-4, 25 × 4 mm, 25 μm
Mobile phase: 2-propanol in water; flow-rate 1 ml/min
Detection: UV, 263 nm

for all three precolumns. This indicates good preconcentration efficiency of the ADS stationary phases for itraconazole and its metabolite.

Selectivity of the sample clean-up step was examined with the precolumns coupled into the column-switching system. The Fig. 3a shows the different behavior of ADS precolumns after injection of the blank serum samples (before treatment of the patient with IC). It is obvious that the ADS RP-4 precolumn seems to be the best choice for the sample preparation step because fewer interfering peaks appear in the chromatogram compared with both the ADS RP-8 and ADS RP-18 precolumns.

Fig 3b shows the different behavior of ADS precolumns during the analysis of a patient sample containing both IC and HIC (serum concentrations 317 ng/ml of IC and 327 ng/ml HIC). Nonsymmetrical peaks of analytes were observed in the column-switching system using both ADS RP-18 and RP-8 precolumns. However, separation using the ADS RP-4 was satisfactory. The asymmetry factors of the IC and HIC peaks grow with increasing alkyl chain length of the packing material, that means with increasing hydrophobicity of the internal surface. The variations in the symmetry of the peaks are presumably caused by differences in the desorption of analytes from the precolumn into the analytical column.

Taking the above factors into consideration, the ADS RP-4 precolumn was chosen for the following analyses and for validation of the column-switching assay.

Itraconazole is a weakly basic compound interacting with free silanol groups of a stationary phase. In previously published papers, acceptable separation has been achieved mainly using mobile phases containing triethylamine/diethylamine [9, 13, 14, 17, 18] or ion-pair agents [1, 16] on C8 or C18 analytical columns.

Three different octylsilica stationary phases were tested: LiChrosorb RP8, Symmetry C8, and Symmetry Shield C8. Chromatographic parameters (capacity ratio, peak asymmetry, and separation efficiency values) are summarized in Table 2.

Various mixtures of methanol and water were tested as mobile phases II. The best resolution of HIC from peaks of serum matrix and the shortest analysis time were obtained using a mixture of methanol : water 75 : 25.

Adequate chromatographic separation was achieved on a LiChrosorb RP8, but the addition of 0.2% triethylamine was necessary to improve the peak asymmetry value. This is in agreement with previously published papers, where the peak tailing linked to silanol activity was minimised by addition of amine modifiers.

The symmetry C8 column, containing a base-deactivated stationary phase, offered excellent peak asymmetry values (As ≈ 1.15–1.2) for both the compounds analysed, IC and HIC, neither amine modifier nor buffered mobile phase being used. Symmetry Shield C8, a column containing a stationary phase with an inverted carbamate group incorporated into the structure, minimises the interactions of itraconazole and its metabolite with surface silanols resulting in further improvement of the peak shape and separation efficiency values (Table 1). When coupling either Symmetry C8 or Symmetry Shield C8 columns with the ADS RP-4 precolumn into the column-switching system, the peak asymmetry values increased to 1.5–1.7 due to desorption from the ADS RP-4 precolumn.

The Symmetry C8 analytical column and ADS RP4 precolumn were chosen for the analysis of clinical serum samples and for validation of the column-switching procedure. No interferences were observed in the chromato-

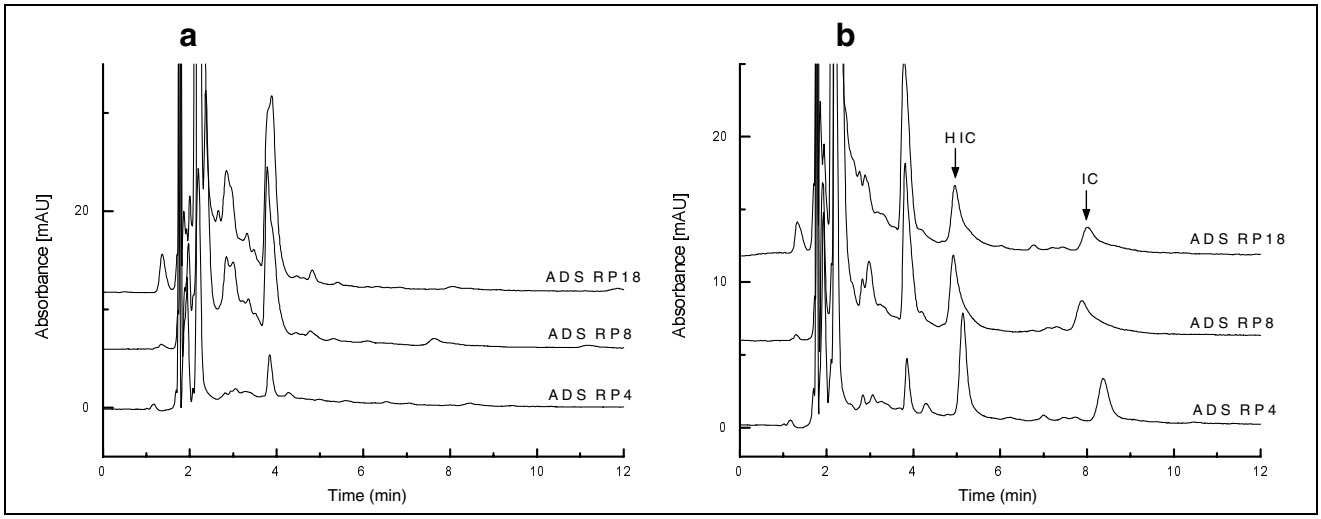

Fig. 3a, b: Chromatographic separations of blank patient serum samples before treatment (**a**) and serum samples during treatment containing IC (317 ng/ml) and HIC (327 ng/ml) (**b**) using different precolumns
Column: Symmetry C8, 150 × 4.6 mm, 5 μm

Table 1: Capacity ratio (k′), assymetry values (As) and number of theoretical plates (n) for IC and HIC on the different C8 analytical columns

Column	IC			HIC		
	k′	As	n (TP/m)	k′	As	n (TP/m)
LiChrosorb RP8[a]	8.1	1.50	21500	4.2	1.40	19500
Symmetry C8[b]	7.3	1.20	32000	3.8	1.15	33000
Symmetry Shield C8[b]	6.8	1.10	39000	3.5	1.05	37500

[a] Mobile phase with addition of 0.2% triethylamine (pH 3.0 adjusted with phosphoric acid)
[b] Mobile phase was methanol : water 75 : 25

Table 2: Timetable of the analysis

Time (min)	Autosampler	Switching valve	Action
0	INJECT	LOAD	injection of 1st sample
7		INJECT	analyte transfer
9		LOAD	reconditioning precolumn
19	INJECT	LOAD	injection of 2nd sample

Table 3: Validation parameters for the column-switching system

Parameter	HIC	IC
Repeatability		
– of retention time[a]	0.43%	1.1%
– of injection[b]		
2.74 μg/ml	1.75%	1.27%
1.37 μg/ml	3.97%	3.91%
137 ng/ml	5.29%	6.80%
Calibration line[c]		
– intercept	1.19 ± 2.80	2.75 ± 3.50
– slope	56.09 ± 0.53	37.41 ± 0.69
– correl. coefficient	0.995	0.991
– F-test[d]	1.27	1.51
LOQ	12 ng/ml	15 ng/ml
Recovery		
2.74 μg/ml	102.3 ± 3.2%	98.7 ± 3.7%
1.37 μg/ml	98.7 ± 4.5%	97.6 ± 4.8%
137 ng/ml	98.5 ± 5.2%	101.8 ± 5.9%

[a] RSD from ten measuremets
[b] RSD from six measuremets
[c] duplicate injection of six calibration standards
[d] Fcrit (0.95;a;a) = 4.53

grams of clinical samples during method development and its application, and no changes in precolumn back-pressure were observed during the measurements which indicates a long life for the ADS materials, as reported in [28].

The analysis time including sample preseparation was 19 min. However, the column switching system (Table 2) allows operation with two samples simultaneously. Therefore at least 5 samples per hour in total could be analysed. Precision was evaluated as the repeatability for six serum samples at three concentration levels. The RSD values, summarized in Table 3, are comparable with (or better than) other published preseparation procedures [1, 9, 14]. The repeatabilities of retention time, calculated as RSD value of 10 injections, show good stability of the column-switching chromatographic system.

The calibration curve was measured from duplicate measurements of six calibration standards in the concentration range from LOQ to 2.7 μg/ml and the dependence of peak area on concentration was evaluated. The intercept and slope and their standard deviations, and correlation coefficients are presented in Table 3. The F-test, based on analysis of variance applied to regression analysis, relates to good linearity of a linear calibration curve.

The relative extraction recoveries were calculated as a ratio between the response of the analyte in the fortified serum sample and the response of the standard solution. The values presented (Table 3) do not differ significantly from 100%. No memory effects were observed during the development and validation of the assay.

Limits of quantitation were calculated from the baseline noise (10 times the baseline noise) and were approximately a magnitude lower (12–15 ng/ml) then the minimum therapeutic concentration levels (> 250 ng/ml [2, 14]). In comparison with previously published assays (LOQs from 6 ng/ml [14, 17] to 45 ng/ml [1]), similar concentration levels could be analysed with higher sample volumes, which were 250–1000 μl of serum sample for the manual sample clean-up assays.

3. Experimental

3.1. Chemicals and reagents

Itraconazole and hydroxy-itraconazole standards were kindly provided by the National Institute of Oncology in Bratislava. Acetonitrile, methanol, 1-

propanol, 2-propanol, water (all of gradient grade), and triethylamine (analytical grade) were purchased from Merck, Darmstadt, Germany, disodium hydrogenphosphate and potassium dihydrogenphosphate dodecahydrate (analytical grade) were purchased from Lachema, Brno, Czech Republic.
The stock solutions of the compounds analyzed were prepared in methanol at concentrations of 0.1 mg/ml. The working solution was prepared by diluting 2.5 ml of stock solution with water in a 10 ml volumetric flask. The calibration standards were prepared by diluting 1000, 500, 250, 100, 50, and 25 µl of working solution with water in 10 ml volumetric flasks. The quality control samples (spiked serum samples) were prepared by diluting the appropriate amount of working solution with blank serum.
50 mM phosphate buffer (pH 7.2) was prepared as follows: 2.042 g of potassium dihydrogenphosphate and 12.535 g of disodium hydrogenphosphate dodecahydrate were dissolved in 1 l of water.

3.2. Chromatographic equipment and conditions

The chromatographic system included HPLC pumps 501 and 510, a 484 UV Variable Wavelength Detector (Waters, Milford, USA), a Basic-Marathon autosampler (Spark, Emmen, The Netherlands) and an AI 015 Automated Switching Valve (DeltaChrom, Watrex, Bratislava, Slovakia). The following analytical columns were used: LiChrosorb RP-8, 250 × 4 mm, 10 µm (Merck, Darmstadt, Germany), Symmetry C8 and Symmetry Shield C8, 150 × 3.9 mm, 5 µm with Symmetry C8 and Symmetry Shield C8 guard columns 20 × 4 mm, 5 µm (Waters, Milford, USA). LiChrospher ADS RP-4, RP-8, and RP-18, 25 × 4 mm, 25 µm (Merck, Darmstadt, Germany) were used as precolumns in the column-switching system.
The washing mobile phase was 50 mM phosphate buffer (pH 7.2), the flow-rate was 1.4 ml/min. Mobile phase II – the eluting mobile phase – consisted of 75% methanol and 25% water and the flow-rate was 0.8 ml/min. The separation was detected at 263 nm.

3.3. Sample clean-up procedure

The serum sample was centrifuged at 3000 min^{-1} for 5 min and a quantity of 100 µl was injected directly into the column-switching chromatographic system (Fig. 1). The timetable for the valve positions is given in Table 2.

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