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Selective high-performance liquid chromatographic assay for itraconazole and hydroxyitraconazole in plasma from human immunodeficiency virus-infected patients

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Abstract

A sensitive and selective reversed-phase liquid chromatographic assay for itraconazole and hydroxyitraconazole in human plasma has been developed and validated. Itraconazole and hydroxyitraconazole were extracted from the matrix using solid-phase extraction on a strong cation-exchange sorbent. All compounds were detected using fluorescence at 265 and 363 nm for excitation and emission, respectively. The assay has been validated over the range 10–1000 ng/ml for both compounds, 10 ng/ml being the lower limit of quantification. Accuracies ranged from 104 to 113% for itraconazole and from 91 to 103% for hydroxyitraconazole. The intra-assay precisions were all below 9% for itraconazole and below 8% for hydroxyitraconazole. The selectivity has been evaluated with respect to all registered anti-human immunodeficiency virus (HIV) drugs and other potential co-medications and a few of their metabolites, commonly used by HIV-infected individuals. Both itraconazole and hydroxyitraconazole were stable under relevant conditions for HIV-inactivation and storage of samples. The applicability of the assay was demonstrated for samples collected from a treated HIV-infected patient.

Keywords: Itraconazole; Hydroxyitraconazole

1. Introduction

Itraconazole (Fig. 1) is an orally administered triazole compound with a broad spectrum of activity against dermatophytes, yeasts (e.g. *Candida* spp. and *Cryptococcus neoformans*), dimorphic and dematiaceous fungi, and some moulds such as *Aspergillus*. It is approved for the treatment or prophylaxis of systemic fungal infections in both immunocompetent and immunocompromised patients [1].

Itraconazole is highly lipophilic and virtually insoluble in water (<1 mg/l). As a weak base (pK_a 3.7) it is ionised at low pH. Itraconazole is widely distributed in the body, with an apparent volume of distribution of 10.7 l/kg after intravenous administration. The drug is 99.8% bound to human plasma proteins, primarily albumin [1]. Itraconazole is extensively metabolised by the hepatic cytochrome

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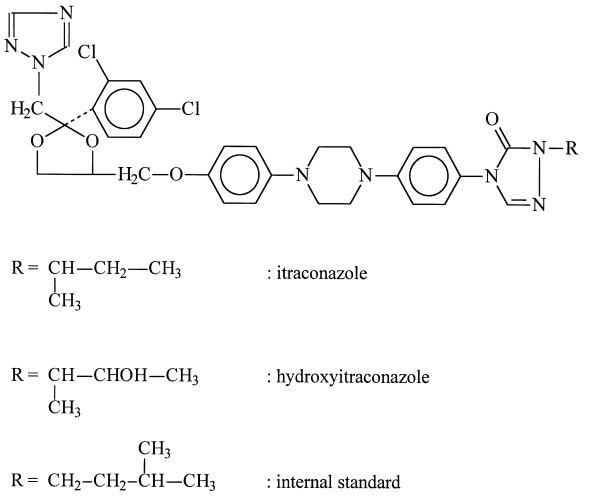

Fig. 1. Chemical structures of itraconazole, its metabolite hydroxyitraconazole and the internal standard (R051012).

P450 isoenzymes. It undergoes side chain hydroxylation, which yields the active metabolite hydroxyitraconazole (Fig. 1). Hydroxyitraconazole reaches plasma concentrations two to three times higher than itraconazole. Most other metabolites that are produced after cleavage of the azole ring are inactive [1,2].

Several biologic and high-performance liquid chromatographic (HPLC) assays for the analysis of itraconazole in plasma and tissue have been described [3–14]. Biologic assays [3–6] have the disadvantage that they are non-specific and unable to distinguish the metabolite from the parent compound. More significantly, hydroxyitraconazole, which is bioactive, contributes four to six times the activity of itraconazole in bioassays [4]. Another disadvantage is that these methods lack adequate precision and sensitivity for therapeutic drug monitoring [3]. HPLC methods have been used more successfully; the first assay was reported in 1987 by Woestenborghs et al. [7]. This is still the most sensitive assay for itraconazole ever reported, however, to obtain a lower limit of quantification (LLQ) of 1 ng/ml, far below any therapeutic range, 2 ml of plasma and five liquid–liquid extraction steps were used. The first method for both itraconazole and hydroxyitraconazole was reported by Poirier et al. [8]; their method using a triple liquid–liquid extraction was modified later into a single extraction [9]. In addition to liquid–liquid extraction [8–10], the simultaneous determination of both analytes can also be obtained using a simple protein precipitation [11–14] or a solid-phase extraction on octadecyl-modified silica [11] as a pre-treatment procedure. In these assays both ultraviolet absorbance [3,7, 10,11,14] and fluorescence detection [5,8,9,12,13] have been used.

In this paper we describe the development and validation of a reversed-phase HPLC assay for itraconazole and its active metabolite hydroxyitraconazole in human plasma after ion-exchange solid-phase extraction (SPE), which can be automated easily with an ASPEC sample processor. The selectivity was tested especially for samples originating from HIV-infected patients using all registered anti-HIV drugs.

2. Experimental

2.1. Chemicals

Itraconazole (R051211), hydroxyitraconazole (R063373) and the internal standard (R051012) originated from Janssen Research Foundation (Beerse, Belgium). The pharmaceutical agents used for testing analytical interference were obtained as pharmaceutical grade reference material or as a solution for injection. Blank, drug-free human plasma was obtained from the Bloedbank Midden Nederland (Utrecht, The Netherlands). Acetonitrile (gradient grade) and methanol (HPLC grade) were provided by Biosolve (Valkenswaard, The Netherlands) and analytical grade triethylamine by Merck-Schuchardt (Hohenbrunn, Germany). Water was home-purified by reversed osmosis on a multi-laboratory scale and all other chemicals were of analytical grade from Merck (Darmstadt, Germany). Phosphate buffer (pH 6.0) was prepared by mixing 0.1 *M* sodium dihydrogenphosphate (900 ml) with 0.1 *M* disodium hydrogenphosphate (135 ml).

2.2. Equipment

Chromatographic analyses were performed using the following equipment. A P580 isocratic pump (Gynkotek HPLC, Germering, Germany), a Basic+

Marathon autosampler (Spark Holland, Emmen, The Netherlands), equipped with a 7739-005 injection valve (Rheodyne, Cotati, CA, USA) with a 100-μl sample loop and an RF-10Ax1 fluorescence detector (Shimadzu, Kyoto, Japan). Data were recorded on a Jotronics Pentium 166-32 Mb personal computer (Delfgauw, The Netherlands), equipped with a Chromeleon chromatographic data system version 4.32 (Gynkotek HPLC).

For SPE, Supelclean™ LC-SCX cartridges (Supelco, Bellefonte, PA, USA) containing 100 mg sulphonic acid modified silica as the sorbent were processed with a 20-port Isolute vacuum manifold (Supelco, Bellefonte, PA, USA). Alternatively, an ASPEC XL sample processor (Gilson, Villiers-le-Bel, France) was used for SPE. Further, a model 5417R centrifuge (Eppendorf, Hamburg, Germany) and a Dri-Block DB-2A (Techne, Duxford, Cambridge, UK) were used.

2.3. Chromatographic conditions

A volume of 50 μl was injected on a Symmetry C_{18} column (100×4.6 mm, d_p=3.5 μm, average pore diameter=10 nm, Waters, Milford, MA, USA), protected by a Symmetry C_{18} pre-column (20×3.8 mm, d_p=5 μm, Waters). The column was operated at ambient temperature. The eluent comprised 55% (v/v) acetonitrile and 45% (v/v) of 0.1% (w/v) triethylamine in water; the aqueous phase was adjusted to pH 3.0 using 85% (w/v) phosphoric acid. The eluent flow-rate was 1.0 ml/min. The fluorescence detection wavelengths were 265 nm for excitation and 363 nm for emission.

2.4. Sample pre-treatment

To 0.5 ml of a plasma sample, pipetted into a polypropylene micro tube, 25 μl of the internal standard solution (3 μg/ml R051012 in methanol) and 75 μl of 3 *M* aqueous hydrochloric acid were added. After vortex mixing for ~30 s and centrifuging at ~10 600 *g* for 5 min, ~500 μ1 of the supernatant was loaded onto a conditioned SPE column. The column was conditioned previously using 1 ml methanol and 2 ml of 1.1% (v/v) perchloric acid in water (pH 1.5). The column was kept wetted throughout the whole procedure. After sample loading and successive washings with 1.0 ml of 0.01 *M* hydrochloric acid in methanol, 1.0 ml of 0.1 *M* phosphate buffer (pH 6.0) and 1.0 ml of distilled water, elution of the analytes was performed with 1.0 ml methanol. The eluate was evaporated to dryness under a nitrogen stream at 40 °C and the residues were reconstituted in 200 μl HPLC eluent by vortex-mixing for ~30 s. Next, the solution was transferred to an injection vial fitted with a glass 250-μl insert for injection.

The solid-phase extraction was also tested on the ASPEC sample processor, operated at ambient temperature. The column was conditioned and washed using a 6-ml/min flow-rate. The sample was loaded at 1 ml/min and the analytes were eluted at 2 ml/min.

2.5. Validation

Four stock solutions of 517 and 502 μg/ml hydroxyitraconazole and 531 and 529 μg/ml itraconazole in methanol were prepared (all with separate weighings) and were stored at −20 °C. For calibration, a dilution of 1034 ng/ml hydroxyitraconazole and 1062 ng/ml itraconazole in plasma pooled from three individuals was made from the 517 μg/ml hydroxyitraconazole and 531 μg/ml itraconazole stock solutions and was also stored at −20 °C. Dilutions of this standard in plasma yielding 10, 21, 52, 103, 207, 517 and 1034 ng/ml hydroxyitraconazole and 11, 21, 53, 106, 212, 531 and 1062 ng/ml itraconazole calibration samples in plasma, respectively, were made daily and analysed for each analytical run. Least-squares linear regression, weighted by X^{-2} (reciprocal of the squared concentration), was employed for the calibration of both hydroxyitraconazole and itraconazole using the ratio ((hydroxy)itraconazole/internal standard) of the heights of the fluorescence peaks. The powers of the weighting factors were determined by maximizing the log-likelihood functions; this statistical procedure was performed using SPSS 7.5 software (SPSS, Chicago, IL, USA).

The 502 μg/ml hydroxyitraconazole and 529 μg/ml itraconazole stock solutions were used to obtain validation (QC, quality control) samples in human plasma at 10, 20, 201 and 753 ng/ml hydroxyitraconazole and 11, 21, 212 and 794 ng/ml it-

raconazole, respectively. These four samples are called QC-LLQ, QC-low, QC-medium and QC-high. Plasma from different individual donors was used. Precisions and accuracies were determined by six-fold analysis of each validation sample in three analytical runs on separate days for all concentrations. Relative standard deviations were calculated for both the intra-day precision (repeatability) and the inter-day precision (reproducibility).

A total of six individual blank plasma samples from different individuals were processed to test the selectivity of the assay. The selectivity of the assay was further tested by investigating the chromatographic response of several pharmaceutical compounds, including some of their metabolites, potentially co-used by HIV-infected patients treated with itraconazole. Reference solutions were prepared from zidovudine, stavudine, didanosine, lamivudine, zalcitabine (nucleoside reverse transcriptase inhibitors), delavirdine, nevirapine, efavirenz (non-nucleoside reverse transcriptase inhibitors), amprenavir, indinavir, saquinavir, nelfinavir, ritonavir (protease inhibitors), pyrimethamine, fluconazole, sulfamethoxazole, trimethoprim, ganciclovir, pyrazinamide, methadone, rifampicin (other drugs commonly used by HIV-infected patients), paracetamol, morphine, oxazepam, ranitidine, folic acid, caffeine (commonly used other drugs), 3′-amino-3′-deoxythymidine and zidovudine-glucuronide (metabolites). Details of these standard solutions have been reported in Table 1. The reference solutions were combined (six mixtures of four or five compounds together; Table 1) and spiked into QC-LLQ samples. For all tested compounds 10 μg/ml in plasma was obtained except for morphine (72 μg/ml), paracetamol (20 μg/ml) and caffeine (15 μg/ml).

Table 1
Reference solutions for selectivity testing

Name	Solvent	Concentration (mg/ml)	Present in mixture no.
3′-Amino-3′-deoxythymidine	Water	1.0	2
Amprenavir	Methanol	0.28	1
Caffeine	Methanol	1.5	6
Delavirdin	Acetonitril/methanol (1/9, v/v)	1.0	5
Didanosine	Methanol	1.0	4
Efavirenz	Methanol	1.0	2
Fluconazole	Methanol	1.0	3
Folic acid	Methanol	1.0	3
Ganciclovir	Methanol	1.0	4
Indinavir	Methanol	0.40	1
Lamivudine	Water	1.0	2
Methadone	Methanol	1.0	3
Morphine	Water	7.2	6
Nelfinavir	Methanol	0.40	1
Nevirapin	Dimethylsulfoxide	1.0	6
Oxazepam	Methanol	1.0	4
Paracetamol	Methanol	2.0	6
Pyrazinamide	Methanol	1.0	5
Pyrimethamine	Methanol	1.0	6
Ranitidine	Methanol	1.0	5
Rifampicin	Methanol	1.0	5
Ritonavir	Methanol	0.40	1
Saquinavir	Methanol	0.40	1
Stavudine	Methanol	1.0	4
Sulphamethoxazole	Methanol	1.0	3
Trimethoprim	Methanol	1.0	4
Zalcitabine	Methanol	1.0	2
Zidovudine	Water	0.50	2
Zidovudine-glucuronide	Methanol	1.0	3

For the determination of the recovery of both compounds, three reference samples were prepared in eluent at the concentrations of validation samples QC-low, QC-medium and QC-high. All these reference samples were measured six-fold in each of three runs together with the QC samples.

The stability of itraconazole and hydroxyitraconazole was investigated for the QC-low and QC-high samples in triplicate under the following conditions: performing three extra freeze–thaw cycles on the QC samples, a 4-h exposure of the plasma samples to ambient temperature, a 1-h exposure of the biological samples to 56 °C in a water bath (leading to inactivation of the HIV) [15], a 3-day storage of the dried extracts at ambient temperature (''in-process'') and a 24-h storage of the final samples in the autosampler.

An HIV-infected patient received 200 mg itraconazole orally (twice daily, at steady state) in addition to anti-HIV drugs. Blood samples were taken at 12 different time points during 12 h for pharmacokinetic evaluation.

3. Results and discussion

Itraconazole, hydroxyitraconazole and the internal standard are weakly basic, highly lipophilic compounds. To obtain acceptable chromatographic results we found that reversed-phase HPLC with a C_{18} column was most suitable. The pH of the aqueous part of the eluent was chosen in the lower range in view of the fluorimetric detection. The intensity of fluorescence depends strongly on the pH of the solvent and increases with decreasing values of pH [16]. Phosphoric acid was used to acidify the eluent to establish a stable pH of the aqueous part and provided for a sufficient buffer capacity. The optimal excitation and emission wavelengths for the three triazole analogues in the eluent were used for the quantification.

Different SPE sorbents were tested (octadecyl, cyanopropyl and aromatic sulphonic acid). Aromatic sulphonic acid, as a strong cation exchanger, bound itraconazole, hydroxyitraconazole and the internal standard that were protonated after addition of 3 *M* hydrochloric acid to a pH<2. Washing occurred consecutively with 0.01 *M* hydrochloric acid in methanol and 0.1 *M* aqueous phosphate buffer at pH 6.0, to elute non-ionic lipophilic compounds and non-lipophilic ionic compounds, respectively. Furthermore, washing was performed with distilled water to remove phosphate ions. The analytes were eluted from the sorbent with 100% methanol. Using octadecyl or cyanopropyl SPE sorbents for extraction, clean chromatograms could also be obtained, however, recovery was lower and reproducibility was poor.

Fig. 2 shows chromatograms of double blank human plasma (no I.S. added), a spiked plasma sample and a patient sample. The retention times of itraconazole, hydroxyitraconazole and the internal standard were 3.9, 10.3 and 15.3 min, respectively.

3.1. Validation

The assay was linear over a concentration range of 10–1000 ng/ml in human plasma, as is shown in Tables 2 and 3; for nine calibration curves the calibration concentrations were back-calculated from the ratio of the peak heights. The deviation of the nominal concentration for all concentrations was equal to or less than 3% (Table 3) without any sign of non-linearity.

Assay performance data are presented in Table 4. At the lower limit of quantification (LLQ, 10 ng/ml) the accuracies and precisions were within the required ±20% range [17,18]. The accuracies and precisions for the other tested concentrations were

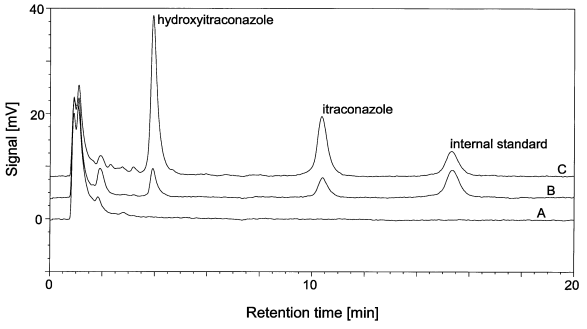

Fig. 2. Chromatograms of (A) double blank human plasma (no I.S. added), (B) a spiked plasma sample containing 103 ng/ml hydroxyitraconazole and 106 ng/ml itraconazole, and (C) a patient sample containing 653 ng/ml hydroxyitraconazole and 361 ng/ml itraconazole.

Table 2
Weighted linear regression parameters of itraconazole and hydroxyitraconazole in the range 10–1000 ng/ml ($n=9$)

Compound	Intercept±SD (10^{-3})	Slope±SD (10^{-3} ml/ng)	Standard error±SD (10^{-3})
Hydroxyitraconazole	4.9±9.5	6.8±0.7	8.9±5.2
Itraconazole	9.2±7.7	5.5±0.5	5.6±3.1

Table 3
Back-calculated concentrations ($n=9$) of hydroxyitraconazole and itraconazole in plasma calibration samples

Nominal concentration (ng/ml)	Concentration found±SD (ng/ml)	Reproducibility (%)	Accuracy (%)
Hydroxyitraconazole			
10.3	10.3±0.4	4	100
20.7	20.9±1.8	8	101
51.7	52.4±3.6	7	101
103	101±6	6	98
207	205±7	3	99
517	520±14	3	101
1034	1039±44	4	100
Itraconazole			
10.6	10.7±0.4	3	100
21.2	20.7±1.3	6	98
53.1	53.7±2.1	4	101
106	17±3	3	102
212	214±6	3	101
531	523±14	3	98
1062	1052±50	5	99

Table 4
Assay performance data for the determination of hydroxyitraconazole and itraconazole ($n=18$)

Nominal concentration (ng/ml)	Repeatability (%)	Reproducibility (%)	Accuracy (%)
Hydroxyitraconazole			
10.0	7	8	98
20.1	5	6	94
201	2	6	103
753	7	8	91
100[a]	11		107
500[a]	8		95
Itraconazole			
10.6	6	9	106
21.2	7	9	104
212	2	6	113
794	2	4	104
100[a]	7		112
500[a]	9		96

[a] SPE was performed using the ASPEC system.

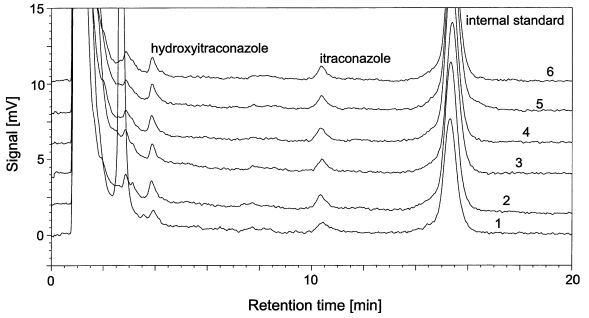

Fig. 3. Chromatograms of plasma spiked with hydroxyitraconazole and itraconazole at the LLQ level and with different combinations of potential interfering compounds (mixtures 1–6, Table 1).

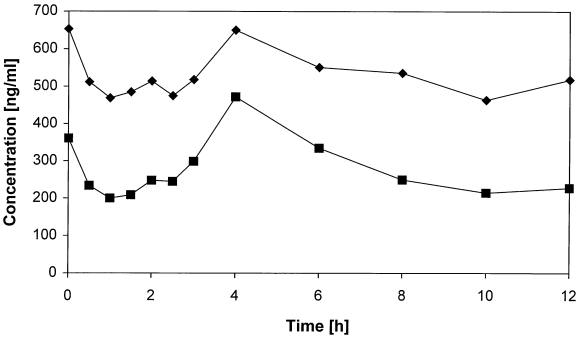

Fig. 4. Pharmacokinetic curve of an HIV-infected patient receiving 200 mg itraconazole twice daily. (■) Itraconazole; (◆) hydroxyitraconazole.

within ±15%. The LLQ is relatively low compared to previously reported values [8–14]. Using the ASPEC, on which the solid-phase extraction was successfully automated, precisions and accuracies remained in the same order of magnitude (Table 4).

The chromatograms of six batches of control plasma contained no endogenous constituents coeluting with either itraconazole, hydroxyitraconazole or the internal standard. All tested potential comedications and metabolites also showed no interference (Fig. 3). No more than three anti-HIV drugs had been tested previously [12]. The high selectivity, from all tested compounds only one extra peak could be observed (Fig. 3, mixture 1), is caused by the extraction with efficient washing steps facilitated by the weak basic and highly lipophilic molecules and by the selective fluorescent properties.

The average recovery obtained for itraconazole was 85%, and was 86% for hydroxyitraconazole. Stability data of the analytes in plasma, extract and final sample are presented in Table 5. No degradation was observed under all conditions tested. Itraconazole and hydroxyitraconazole were already shown to be stable in plasma for 6 months at −20 °C and for 1 year at 4 °C in methanol [10].

Finally, the pharmacokinetic curves of itraconazole and its metabolite in an HIV-infected patient are demonstrated in Fig. 4 and show that the assay may be applied for the analysis of plasma from HIV-infected individuals.

4. Conclusion

For the quantification of itraconazole and its active metabolite hydroxyitraconazole, a selective and sensitive HPLC assay meeting all common validation criteria was developed. Both compounds are stable

Table 5
Stability data (n=3) of hydroxyitraconazole and itraconazole, recovery±SD (%)

Conditions	Hydroxyitraconazole		Itraconazole	
	21 ng/ml	794 ng/ml	20 ng/ml	753 ng/ml
Three extra freeze–thaw cycles (plasma)	80±9	87±3	80±6	97±4
4-h ambient (plasma)	93±9	80±9	115±9	102±6
1-h 56 °C (plasma)	96±5	86±7	101±1	96±3
3-day ambient (extract)	88±7	90±2	108±7	103±2
24-h ambient (final samples)	89±7	88±7	114±5	101±6

under all conditions tested. The assay can be used for HIV-infected and other patients in the future.

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