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Liquid chromatographic determination of ketoconazole, a potent inhibitor of CYP3A4-mediated metabolism

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Abstract

A high-performance liquid chromatographic assay with UV detection has been developed for the determination of ketoconazole in human plasma. Quantitative extraction was achieved by a single solvent extraction involving a mixture of acetonitrile–*n*-butyl chloride (1:4, v/v). Ketoconazole and the internal standard (clotrimazole) were separated on a column packed with Inertsil ODS-80A material and a mobile phase composed of water–acetonitrile–tetrahydrofuran–ammonium hydroxide–triethylamine (45:50.2:2.5:0.1:0.1, v/v). The column effluent was monitored at a wavelength of 206 nm with a detector range set at 0.5. The calibration graph was linear in the range of 20–2000 ng/ml, with a lower limit of quantitation of 20.0 ng/ml. The extraction recoveries for ketoconazole and clotrimazole in human plasma were 93±9.7% and 83±10.0%, respectively. The developed method has been successfully applied to a clinical study to examine the pharmacokinetics of ketoconazole in a cancer patient.

Keywords: Ketoconazole

1. Introduction

There is considerable motivation for understanding drug–drug interactions with anticancer agents because of their narrow therapeutic index and the numerous concomitant medications that are administered routinely or intermittently [1]. These interactions may arise as a result of altered pharmacodynamics or pharmacokinetics of the drugs involved. In the latter case, this is usually due to modification of tissue distribution and metabolism of the drugs. Most of the data currently available to evaluate potential drug interactions with (novel) anticancer agents have been addressed by animal experiments or the use of test systems in vitro [1]. It has been shown that ketoconazole (Fig. 1), a synthetic imidazole-type broad-spectrum antifungal agent is a highly potent inhibitor of CYP3A4 [2,3], and can inhibit anticancer drug metabolism at concentrations as low as 1 μM [2,4]. Previous investigations have shown that with standard clinical doses of ketoconazole (200–400 mg/day orally), peak plasma concentrations are in the range of 4 to 20 μM [5], suggesting that concomitant treatment of ketoconazole is likely to substantially alter the disposition of various anticancer agents when administered to patients.

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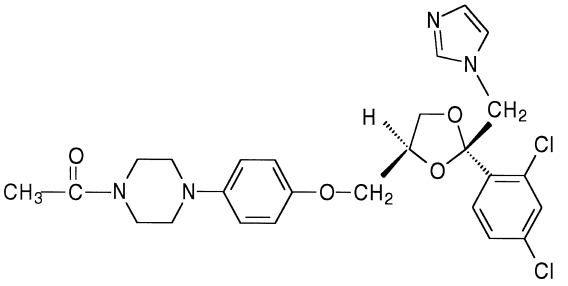

Fig. 1. Chemical structure of ketoconazole.

Although the key rationale for conducting experiments in vitro on metabolism and drug–drug interactions is the presumed applicability to the clinical situation, for most anticancer agents there are no data yet indicating that the in vitro studies are predictive of results in vivo.

Although ketoconazole is generally well absorbed after oral administration, there is large inter- and intra-subject variation in peak concentrations after the same oral dose [6]. It has been proposed, therefore, that determination of ketoconazole concentrations in patients is expedient before attempting to draw any correlation between ketoconazole dose and biological effects. Hence, to enable further investigation of the interaction between ketoconazole and anticancer agents, a specific and accurate assay method for the determination of ketoconazole in human plasma was considered obligatory. Several microbiological [7–9], spectrofluorimetric [10] and electrochemical assays [8], as well as high-performance liquid chromatography (HPLC) methods [11–16] have been described for the determination of ketoconazole in human plasma (using UV or fluorescence detection), but generally, these lack sufficient sensitivity. The reported HPLC methods offer greater precision than the microbiological assays, but are no more sensitive and involve complicated (solid-phase) extraction procedures. In this report, we describe a novel HPLC method with UV detection for the determination of ketoconazole in human plasma, with a lower limit of quantitation of 20 ng/ml. The method involves a rapid and highly selective one-step solvent extraction, which avoids the use of expensive and time consuming solid-phase extraction techniques for sample clean up. The method has been extensively validated, and a pilot pharmacokinetic study in a cancer patient receiving ketoconazole was included to investigate the suitability of the method.

2. Experimental

2.1. Chemicals

Ketoconazole (lot 79H4087) and the internal standard clotrimazole (lot 118H1359) were obtained from Sigma (St. Louis, MO, USA). Acetonitrile was obtained from Biosolve (Valkenswaard, The Netherlands). Tetrahydrofuran was obtained from Rathburn (Walkerburn, UK) and triethylamine and ammonium hydroxide from Baker (Deventer, The Netherlands). All chemicals and solvents were of analytical grade or better. Water was purified and deionized by the Milli-Q-UF system (Millipore, Milford, MA, USA). Human plasma was obtained from healthy volunteers via the Blood Bank of the University Hospital Rotterdam (Rotterdam, The Netherlands).

2.2. Preparation of stock solutions

Stock solutions of ketoconazole (1.0 mg/ml) and clotrimazole (1.0 mg/ml) were prepared by dissolving the appropriate amount of drug in acetonitrile and were then stored in glass at −80°C. An internal standard working solution of 10 000 ng/ml was prepared by dilution with a mixture of acetonitrile–water (1:1, v/v), and was stored for later use at 4°C.

2.3. Sample pretreatment

A volume of 100 μl of methanol–0.05 *M* sodium hydroxide (40:60, v/v), 100 μl of internal standard solution (10 μg/ml) and 5 ml of acetonitrile–*n*-butyl chloride (1:4, v/v) were added to 0.5 ml of human plasma in a 12-ml glass tube and closed with a PTFE-faced screw cap. The tube was mixed for 5 min on a multi-tube vortex mixer and centrifuged for 5 min at 4000 *g*. After centrifugation, the organic layer was transferred to a clean glass tube with a Pasteur pipette, and evaporated to dryness under a constant stream of nitrogen at 60°C. A volume of 125 μl of acetonitrile–water (1:1, v/v) was added to the residue, which was reconstituted by ultrasonication for 2 min. After additional centrifugation for 2

min at 4000 *g*, the contents of the tube was transferred to a low-volume glass insert and a 100-μl aliquot was subjected to chromatography.

2.4. HPLC analysis

The chromatographic system consisted of a ConstaMetric 3200 pump (LDC Analytical, Riviera Beach, FL, USA), a Waters 717plus autosampler operating at 4°C (Milford, MA, USA), a Model SpH99 column oven (Spark Holland, Meppel, The Netherlands) and a Spectra Physics UV-2000 detector (San Jose, CA, USA). The stationary phase was composed of Inertsil ODS-80A material (5 μm particles) packed in a 150×4.6 mm I.D. stainless steel column (GL Science, Tokyo, Japan), protected by a LiChrospher 100 RP-18 guard column (4.0×4.0 mm, 5 μm particles). The mobile phase consisted of water–acetonitrile–tetrahydrofuran–ammonium hydroxide–triethylamine (45:50.2:2.5:0.1:0.1, v/v), with the pH adjusted to 6.0 (formic acid). The flow-rate of the mobile phase was set at 1.00 ml/min, and the column effluent was monitored at an absorption wavelength of 206 nm, and a detector range of 0.5. The column temperature was maintained 30°C. Acquisition and integration of data were performed with the ChromCard data analysis system connected to an ICW chromatographic work station (Fisons, Milan, Italy) running on an IBM-compatible computer under Microsoft Windows v95. Calibration graphs were calculated by weighted ($1/x^2$) least-squares linear regression analysis of the peak area ratio of ketoconazole and the internal standard versus the drug concentration of the nominal standard using the Excel v97 software package.

2.5. Precision and accuracy

Method validation was performed according to procedures described in detail by Shah et al. [17]. With each chromatographic validation run, duplicate calibration standards were prepared in blank human plasma by serial dilution at ketoconazole concentrations of 20.0, 50.0, 100, 500, 1000 and 2000 ng/ml. Sets of quality control (QC) samples were prepared in batch in the same manner at 75.0, 750, 1500 and 15 000 ng/ml and were analyzed in quintuplicate. The QC sample containing the highest concentration was used to investigate the effect of sample dilution and/or limited sample-volume injection. The complete validation procedure was performed on 4 separate days. The precision of the assay was assessed by the between-run and within-run precision. Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as classification variable. The between-groups mean square (MS_{bet}), the within-groups mean square (MS_{wit}), and the grand mean (GM) of the observed concentrations across run days were calculated using the NCSS package (Version 5.X, Dr. Jerry L. Hintze, East Kaysville, UT, USA; 1992). The between-run precision (BRP) was defined as:

$$\mathrm{BRP} = \{[(\mathrm{MS}_{\mathrm{bet}} - \mathrm{MS}_{\mathrm{wit}})/n]^{0.5}\} \cdot 100\% \quad (1)$$

where n represents the number of replicates within each validation run. The within-run precision (WRP) was calculated as:

$$\mathrm{WRP} = [(\mathrm{MS}_{\mathrm{wit}})^{0.5}/\mathrm{GM}] \cdot 100\% \quad (2)$$

2.6. Lower limit of quantitation

For determination of the lowest standard concentration in the analytical run (LLQ) with acceptable accuracy and precision, five plasma samples from five individuals were spiked at a ketoconazole concentration of 20.0 ng/ml and analyzed in quadruplicate. For the concentration to be acceptable, the percentage deviation from the nominal value of at least 80% of the samples assayed should be ≤20%, with a resulting WRP of ≤20%.

2.7. Extraction recovery

The extraction recovery of ketoconazole was established at concentrations of 20.0, 50.0, 100, 500, 1000 and 2000 ng/ml, by comparing peak heights of samples prepared in human plasma with those prepared in a mixture of acetonitrile–water (1:1, v/v). The recovery was determined in four independent analytical runs, and expressed as a percentage.

2.8. Specificity and selectivity

Five different human plasma samples were used to investigate the potential interference of endogenous components. In addition, the following commonly used drugs in clinical oncology were used for potential interference with the analytical method: acetaminophen, alizapride, atropine sulfate, codeine, dexamethasone, leucovorin, loperamide, lactulose (Legendal), lorazepam, metoclopramide, morphine hydrochloride, omeprazole, paroxetine, ranitidine and temazepam.

2.9. Stability

The stability of ketoconazole in human plasma was investigated by analysis after storage of the various QC samples at 37°C for 30 h and after three consecutive freeze–thawing cycles. After the indicated duration and storage conditions, the samples were analyzed immediately as outlined above.

2.10. Pharmacokinetic analysis

The patient studied was a 53-year-old male with a histologically confirmed diagnosis of advanced colorectal carcinoma refractory to therapy with 5-fluorouracil. The patient received chemotherapeutic treatment with irinotecan (CPT-11), and during one of the treatment courses, ketoconazole was administered orally at a dose of 200 mg (Nizoral tablets, provided by Janssen-Cilag, Tilburg, The Netherlands), and was given every 24 h for a period of 3 days. The current experiment was approved by the Rotterdam Cancer Institute Review Board, and the patient signed informed consent before study entry.

A total of 16 blood samples (5 ml each) were obtained and collected in 10-ml glass tubes containing 143 units of lithium heparin as anticoagulant. The samples for ketoconazole and analysis were taken immediately before intake and 1, 1.15, 1.3, 1.5, 2, 3, 3.5, 4.5, 6.5, 7.5, 10, 26, 34, 50 and 68 h after dosing. Blood was immediately processed to plasma by centrifugation for 5 min at 3000 *g*, and was kept at −20°C until storage at −80°C. Individual plasma concentration–time data of ketoconazole were analyzed by non-compartmental models using the Siphar version 4.0 software (SIMED, Créteil, France).

3. Results and discussion

3.1. Chromatography

Fig. 2 displays chromatograms of an extract of a blank human plasma sample (A), an extract of a plasma sample spiked with ketoconazole at a concentration of 2000 ng/ml (B), and an extract of a plasma sample taken 6.5 h after ketoconazole intake and an intravenous (i.v.) infusion of CPT-11 (100 mg/m^2) (C). Ketoconazole ($t_R = 5.9$ min) and the internal standard clotrimazole ($t_R = 22.0$ min) were well separated, and the overall chromatographic run time was established at 30 min. Several different drugs were tested for potential interference with ketoconazole and the internal standard (see Experimental), and none of these drugs was found to give an interfering peak during the analysis around the retention time of ketoconazole or the internal standard.

3.2. Validation characteristics

The assay for ketoconazole analysis in plasma was found to be linear over the range of 20 to 2000 ng/ml, applying the peak height in combination with a weighting factor of $1/x^2$, as indicated by the mean linear-regression correlation coefficient of 0.9975 ($n = 4$). In order to increase the sensitivity of the method, the range of the detector was set at 0.5 instead of 1.0. In blank human plasma spiked with ketoconazole at 20 ng/ml, three out of 20 samples were outside the acceptable ±20% deviation limits, while the remaining samples were within 18%, with a mean percentage deviation from the nominal concentration and a within-run variability of 1.7 and 6.6%, respectively. Based on these results, the LLQ was established at 20 ng/ml, which is about three to four times more sensitive than earlier described methods based on HPLC [11–16].

Validation data of the analytical method in terms of accuracy (percent deviation) and precision are shown in Table 1. At the upper limit of quantitation, i.e., 2000 ng/ml, the mean percentage deviation and the within-run variability were also less than 20%. The method was shown to be accurate, with an average accuracy at the four tested concentrations between −5.1 and 11.0%, and precise with a within-

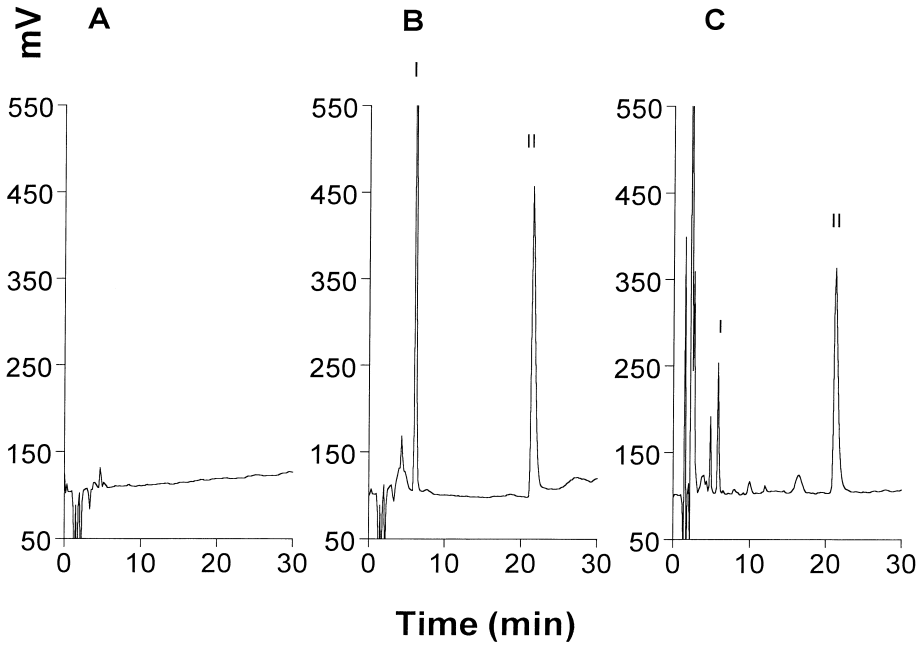

Fig. 2. Chromatograms from reversed-phase HPLC analysis of a blank human plasma sample (A), a human plasma sample spiked with ketoconazole at a concentration of 2000 ng/ml (B), and a plasma sample obtained from a male patient with colorectal cancer at 6.5 h after oral administration of ketoconazole (200 mg) (C). The labeled chromatographic peaks indicate ketoconazole (I) and the internal standard clotrimazole (II), respectively.

run and between-run precision always within 15%. In addition, sample dilution and/or limited sample-volume injection had no effect on the validation characteristics (Table 1). The mean overall extraction recoveries, determined in four separate analytical runs, were 93±9.7% for ketoconazole ($n=45$) and 83±10.0% for the internal standard ($n=45$), and were independent of the spiked concentration. Repeated freeze–thawing cycles had no influence on the stability. In addition, plasma samples spiked with ketoconazole stored for 30 h at 37°C were also stable (not shown).

Table 1
Accuracy, within-run and between-run precision for the analysis of ketoconazole in spiked human plasma samples[a]

Nominal (ng/ml)	GM (ng/ml)	ACC (%)	WRP (%)	BRP (%)	*n*
20.0	20.3	1.7	6.6	7.1	5
75.0	71.2	−5.1	10.4	0.44	5
750	739	−1.5	6.3	2.0	5
1500	1534	2.3	3.9	3.3	5
15 000	16 653	11.0	2.6	2.9	5

[a] Abbreviations: GM, grand mean; ACC, accuracy (percent deviation from nominal value); WRP, within-run precision; BRP, between-run precision; *n*, number of replicate observations within each validation run.

3.3. Clinical pharmacokinetics

The described analytical method was applied to a pharmacokinetic pilot study of ketoconazole given orally to a cancer patient. The observed concentration–time profile of ketoconazole is shown in Fig. 3. The peak concentration of ketoconazole was 4.63 μg/ml (i.e., 8.71 μ*M*), and the area under the concentration–time curve amounted to 12.9 μg h/ml, which is similar to a mean (±SD) value of 12.3±7.7 μg h/ml previously obtained in nine adults with hematological malignancies who were also given a dose of 200 mg [18]. The concentration–time data indicated that the ketoconazole concentration required to completely inhibit CYP3A4-mediated in vitro metabolism of, for example, CPT-11

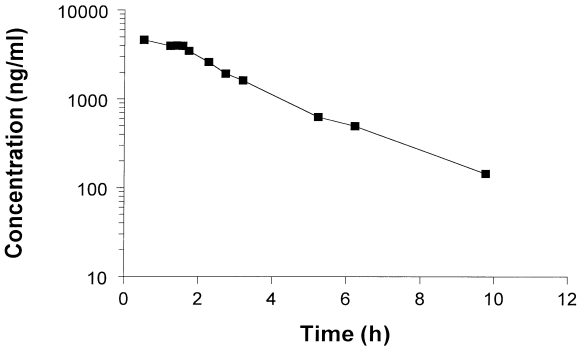

Fig. 3. Plasma concentration–time profile of ketoconazole (– ■ –) after oral administration of a dose of 200 mg to a male cancer patient with colorectal cancer.

(i.e., 1 μ*M*), was achieved for at least 6 h in the studied patient.

In conclusion, the method presented for the determination of ketoconazole in human plasma is specific, accurate and precise, and is selective and sensitive enough to be used in clinical trials. The method permits the analysis of patient samples with low concentrations of ketoconazole, and is currently being used to investigate whether concomitant treatment of CPT-11 with ketoconazole could reduce CYP3A4-mediated drug inactivation in colorectal cancer patients.

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