An HPLC assay for the determination of ketoconazole in common pharmaceutical preparations

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An HPLC method is described using octadecylsilica (3 μm) with an acetonitrile phosphate buffer mobile phase containing diethylamine which is capable of separating ketoconazole [(±)-*cis*-1-acetyl-4-(4{[2-(dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)piperazine] from four related compounds, (R049223, R063600, R053165 and R039519) and from excipients in tablets, cream and shampoo. The method was validated using an external calibration method for tablets, shampoo and creams and a standard addition method for cream. The limits of detection for the related compounds in the presence of ketoconazole are also reported.

Introduction

Ketoconazole [(±)-*cis*-1-acetyl-4-(4{[2-(dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)piperazine] is a broad spectrum antifungal agent used in the treatment of superficial and systemic fungal infections. Formulations of ketoconazole include tablets, creams and shampoo. Four related impurities are known to be associated with ketoconazole: (±)-*cis*-1-acetyl-4-(4-{[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)1,2,3,4-tetrahydropyrazine (R049223), (±)-*cis*-1-acetyl-4-[4-[5-(4-acetyl-1-piperazinyl)-2-{[2-(2-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenoxy]phenyl]piperazine (R063600), (±)-*cis*-1-acetyl-4-[4-{[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]-methoxy}phenyl]piperazine (R053165) and (±)-*cis*-1-[4-{[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl]piperazine (R039519).[1]

It was required, in the course of an ongoing investigation[2] into the quality of drugs available in developing countries, to assay samples of such ketoconazole formulations for active ingredient. Many methods, involving a variety of different analytical techniques, have been published for the assay of ketoconazole.[3–23] Relatively few of these are for the assay of pharmaceutical preparations and in all of these published methods[3,6,10,13,14] the emphasis is on freedom from interference by formulation excipients. Validation is, in general, inadequate to substantiate selectivity with respect to accepted related impurities. Such selectivity is of importance in ensuring accuracy of determination of active ingredient. It is also of potential use in attributing possible causes for any lack of conformity of such preparations with pharmacopoeial limits if particular impurities can be located.

The few liquid chromatography methods[4,7,8] located in the literature for the determination of ketoconazole in pharmaceutical formulations have not addressed the issue of selectivity with respect to related compounds. The pharmacopoeial method[1] for the assay of ketoconazole in tablets specifies only adequate resolution from the internal standard used (terconazole) and a separate thin-layer procedure is specified in the tablet monograph to detect related impurities. In addition, the extraction method used involves a methanol–methylene chloride mixture which is subsequently used as the solvent for sample injection. Use of such a chromatographically strong solvent relative to the methanol in aqueous buffer mobile phase used is likely to degrade the efficiency of the resultant chromatography appreciably. A more recent publication[8] reports a UV spectrophotometric assay and a modification of the liquid chromatographic pharmacopoeial method. In that publication different mobile phases and extraction procedures are used to those in the pharmacopoeial procedure and the authors comment that no interferences are present in the spectrophotometric procedure.

The objective of the present work was to develop and validate an assay for the determination of ketoconozole formulated as tablets, cream and shampoo which could be demonstrated to be selective with respect to both formulation excipients and also with respect to the four accepted impurities shown in Fig. 1.

Experimental

Equipment and materials

The HPLC equipment used consisted of a Jasco (Great Dunmow, Essex, UK) system comprising a PU-975 intelligent UV/VIS detector operated at 232 nm, a PU-980 intelligent HPLC pump giving a flow rate of 1.5 $cm^3\ min^{-1}$ and an AS-950 intelligent autosampler (20 μl). The HPLC column (200 × 4.6 mm id) was slurry-packed in the laboratory with 3 μm Hypersil ODS supplied by Shandon (Runcorn, UK). Peak area data were collected using a Borwin Chromatography Software package supplied by Jasco. The mobile phase comprised 60% acetonitrile in 20 mM disodium hydrogen orthophosphate containing 0.2% v/v diethylamine, at pH 4.0.

Water was purified by a Millipore (Watford, Herts., UK) Milli Q system. Methanol and acetonitrile were obtained from Rathburn (Walkerburn, UK) and disodium hydrogen orthophosphate, diethylamine and orthophosphoric acid from Fisons (Loughborough, UK). Ketoconazole and its related impurities were kindly donated by Janssen Pharmaceutica. Samples of creams, tablets and shampoo containing ketoconazole were purchased at several different retail outlets in two major cities in Nigeria.

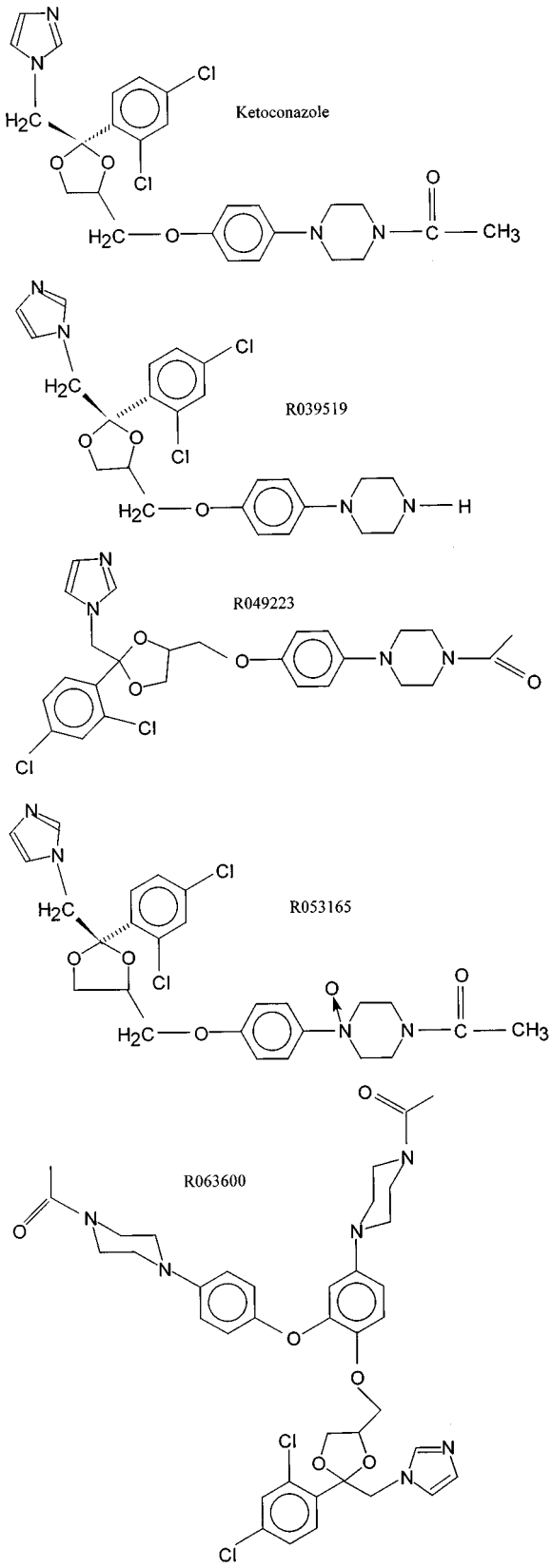

Fig. 1 Structure of ketoconazole and related impurities. Ketoconazole, [(±)-cis-1-acetyl-4-(4{[2-(dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)piperazine]; R039519, (±)-cis-1-[4-{[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]-methoxy}phenyl]piperazine; R049223, (±)-cis-1-acetyl-4-(4-{[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}-phenyl)1,2,3,4-tetrahydropyrazine; R053165, (±)-cis-1-acetyl-4-[4-{[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]-methoxy}phenyl]piperazine; and R063600, (±)-cis-1-acetyl-4-[4-[5-(4-acetyl-1-piperazinyl)-2-{[2-(2-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}-phenoxy]phenyl]piperazine.

Sample preparation

Tablets. Five tablets were chosen at random from each batch of 200 mg tablets. These were weighed and powdered. A quantity equivalent to approximately 200 mg was transferred to a 100 cm^3 volumetric flask and shaken with 70 cm^3 of methanol. After ultrasonification for 30 min the extract was cooled and the volume adjusted to 100 cm^3 with methanol. This was filtered using Whatman (Maidstone, Kent, UK) No. 1 filter paper. A 0.25 cm^3 aliquot was added to 4 cm^3 methanol in a 10 cm^3 volumetric flask and made up to volume with water such that the overall concentration of methanol was 40% v/v and anticipated analyte concentration 50 μg cm^{-3}. The composition of the final solvent being chromatographically weaker than the mobile phase served to maintain minimum peak broadening. Four replicates of the resulting solution (20 μl) were injected for analysis using the external standard calibration line.

Creams. A 1 g sample of cream (20 mg g^{-1}) equivalent to approximately 20 mg ketoconazole was transferred into a 100 cm^3 volumetric flask, weighed and extracted as described above yielding an anticipated analyte concentration of 200 μg cm^{-3}. This solution was then diluted similarly to the tablet extract for application of the external standard method. To carry out the standard addition method a 2 cm^3 sample of this solution was pipetted into each of five 10 cm^3 volumetric flasks. To these were added 0, 1, 2, 3 and 4 cm^3, respectively, of the approximately 200 μg cm^{-3} ketoconazole stock standard. The resulting solutions were each injected (20 μl) four times and the mean peak area used to determine the ketoconazole concentration.

Shampoo. A volume equivalent to 20 mg ketoconazole was quantitatively transferred to a 100 cm^3 flask and extracted as above. The resulting solution was diluted similarly to the tablets to give a ketoconazole concentration of 50 μg cm^{-3} and four replicates of the resulting solution were injected (20 μl) for analysis using the external standard calibration line.

Standard preparation and external standard calibration

The ketoconazole stock standard was prepared by dissolving approximately 10 mg of ketoconazole in 50 cm^3 methanol to achieve a concentration of approximately 200 μg cm^{-3}. Stock solutions of the known impurities were similarly dissolved and diluted to contain approximately 100 μg cm^{-3}. Working standards containing approximately 20, 40, 60 and 80 μg cm^{-3} of ketoconazole were prepared by dilution into water from the stock standard. Each solution also contained 40% v/v methanol to ensure complete solution. A standard calibration of peak area as a function of concentration was prepared by injecting each standard solution four times onto the chromatographic system and obtaining the linear regression of the mean peak area for each standard on concentration.

Results and discussion

Chromatography

A similar rationale for separation to that previously published[15] was used. Octadecylsilica was used as stationary phase with a mobile phase consisting of aqueous phosphate buffer at an optimised pH containing an appropriate proportion of acetonitrile as organic modifier. In the present system diethylamine was employed as a low molecular weight amine to mask residual silanols. The pH was found to have a marked effect on the separation of ketoconazole and its impurities. Fig. 2 shows

the variation in retention time with pH. Increasing pH over a range from 3 to 7 resulted in a general increase in retention and also in significant changes in selectivity among the analytes. Optimum separation occurred at pH 4. Higher pH values resulted in loss of peak symmetry as well as decreased selectivity between analytes. The mobile phase was also optimised with respect to acetonitrile concentration and ionic strength of buffer. To eliminate possible inaccuracies due to matrix interference a standard addition procedure was used to verify the absence of interference from the cream matrix.

Fig. 3(a) shows a typical chromatogram obtained using the optimised mobile phase with an aqueous mixture of ketoconazole and the four related compounds. The resolution between all pairs of compounds is greater than 1.25 and the maximum retention time is in the region of 5 min with the active ingredient of the preparations eluting at 4.2 min. Fig. 3(b), (c) and (d) show representative chromatograms obtained from tablets and creams and shampoo respectively. Only in the chromatogram of cream extract is there any indication of co-extracted compounds. At a retention time of 3.3 min this does not correspond to any of the related impurities and is attributed to cream excipients.

Quantification

Linearity. Calibration equations for the external calibration method were established by regression of peak area on standard concentration for the set of aqueous standards. Typical values of slope following repeated calibration over a five day period ranged from 1215 to 1248. Table 1 shows a mean value of 1236 with a relative standard deviation of 1.017% based on ten calibrations. This indicates good day-to-day precision. Correlation coefficients were in excess of 0.9998. The mean intercept of the external calibration lines at a value of 210 represents 0.49% of the normal analyte response and is thus well within the 2% suggested for adequate validation. The high relative standard deviation of the intercept at 78% is a consequence of the small mean value and does not adversely affect the linearity of the calibration.

Typical values of slope from the standard addition procedure ranged from 1186 to 1232 with a mean value shown in Table 1 of 1205 and relative standard deviation of 1.102%. Correlation coefficients for the standard addition lines were greater than 0.9985. Table 1 also lists the mean and relative standard deviation of the intercept (on the peak area response axis). This corresponds to the response of the analyte extract before standard additions are made. The precision of this, based on five replicates, is seen to be good at 1.27% RSD.

Accuracy. To establish the accuracy of the external calibration method, the peak areas obtained by injecting ketoconazole solutions of known concentration were used to calculate derived concentrations from the calibration regression equation. These derived values were regressed on the known concentration values. Neither the slope (1.027) nor the intercept (-0.16×10^{-3}) of the resultant line were significantly different from one or zero respectively. It was also found that the slopes of the external calibration and standard additions lines were not significantly different ($P = 0.050$) further indicating the absence of interference from components of the cream. The

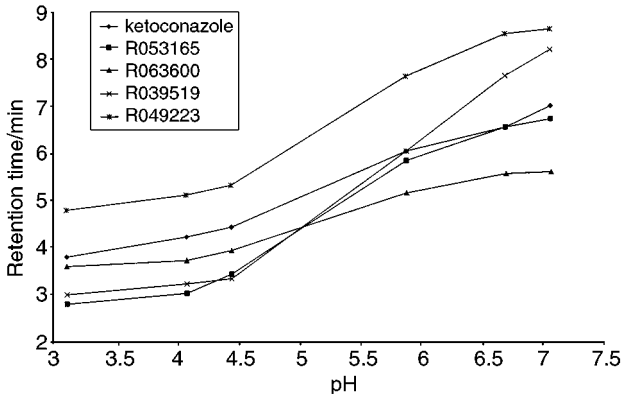

Fig. 2 Variation of retention times of ketoconazole and impurities with pH of mobile phase. Mobile phase consists of 60% acetonitrile in 20 mM disodium hydrogen orthorphosphate containing 0.2% diethylamine.

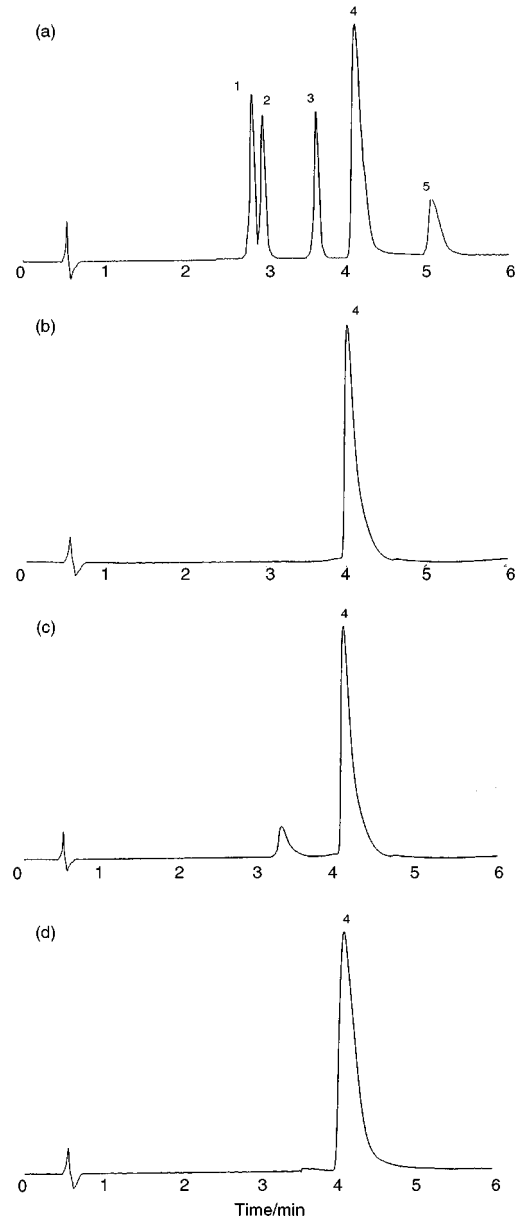

Fig. 3 Typical chromatograms of (a) ketoconazole and related impurities in aqueous solution. Concentration of all solutes 20 μg cm^{-3}; (b) (c) and (d) represent tablet, cream and shampoo extracts, respectively, prepared as described in the text. Compound identification: 1, R053165; 2, R039519; 3, R063600; 4, ketoconazole; and 5, R049223. Mobile phase consists of 60% acetonitrile in 20 mM disodium hydrogen orthophosphate, containing 0.2% diethylamine and adjusted to pH 4 with concentrated orthosphoric acid.

Table 1 Linearity and day-to-day precision of calibration

	n	Mean slope (RSD %)	Mean intercept (RSD %)	Mean r^2 (RSD %)
External standard	10	1236 (1.017)	210 (78.7)	0.9999 (0.007)
Standard addition	5	1205 (1.102)	42762 (1.27)	0.9998 (0.010)

Table 2 Recoveries from spiked dosage forms

Tablet		Cream		Shampoo	
Conc. spiked/ μg cm^{-3}	Mean % recovered (n = 5)	Concn. spiked/ μg cm^{-3}	Mean % recovered (n = 5)	Conc. spiked/ μg cm^{-3}	Mean % recovered (n = 5)
12.65	102.9	25.3	100.2	12.65	103.9
25.3	98.7	50.6	99.7	25.3	101.9
—	—	75.9	102.1	—	—

Table 3 Within-day repeatability

	Aq. standard	Tablet	Cream	Shampoo
Concentration/μg cm^{-3}	62.1	52.4	49.2	51.7
RSD (%) (n = 10)	0.24	0.88	1.24	0.67

Table 4 Detection limits for related impurities

	R053165	R063600	R039519	R049223
LOD/ng cm^{-3}	50	60	80	100
LOD/% ketoconazole (50 μg cm^{-3})	0.1	0.12	0.16	0.2

accuracy of the method was verified by analysing before and after spiking extracts of each dosage form and calculating the drug recovered as a percentage of the amount added. These recoveries are listed in Table 2.

Precision. The within-day repeatability was determined by injecting replicates of an aqueous standard containing approximately 60 μg cm^{-3} ketoconazole and also separate extracts of tablet, cream and shampoo samples following the extraction procedures described above. The relative standard deviations are shown in Table 3.

The relative standard deviation of repeated injections during the course of one day is below 1.25% for any of the preparations chosen. The method is therefore seen to be adequately precise even in the absence of an internal standard that appeared to be a requirement of previously published assays.

Selectivity. The specimen chromatograms shown indicate that the proposed procedure is selective for ketoconazole in the presence of the four related compounds. There is no evidence of excipient interference in the chromatograms of tablet or shampoo extracts. The application of the standard addition method to the assay of ketoconazole in creams and the correspondence of the slopes obtained by both the external standard and the standard addition methods indicates further the selectivity of the chromatography.

Detection limits. The detection limits were determined by injecting successively more dilute solutions of each solute and calculating the resultant signal to noise ratios. The detection limit was recorded as the concentration producing a signal to noise ratio of 3. The value shown in Table 4 for ketoconazole is well below that required for assay of the dosage forms. Table 4 shows the detection limits for the related compounds both as the concentration detectable under the conditions used for the assay of ketoconazole and as a percentage of ketoconazole. These latter values were obtained by determining the limit of detection as above in the presence of a fixed ketoconazole concentration of 50 μg cm^{-3}.

Assay results of dosage forms

Samples of tablets, creams and shampoo were assayed by the method described. Of 18 different tablet samples all were found to be within the British Pharmacopoeial limits of 92.5–107.5% of the stated dose. The amounts found ranged from 93.7 to 107.5% and standard deviations of replicates ranged from 0.1 to 1.2%. Five different cream samples were assayed and a range of content from 84.7 to 93.7% of stated dose found; only one of these falling within the pharmacopoeial limits of 90–110%. Standard deviations on replicate cream samples ranged from 0.1 to 1.3%. Two shampoo samples assayed at 103.6 ± 0.3 and 103.6 ± 0.2, respectively.

Although the method has been demonstrated as being capable of detecting the established related impurities at low concentrations relative to the active drug none of these was detected in any of the samples examined. In the context of the overall investigation outlined in the Introduction, this indicates that the active ingredient used in the dosage forms tested was of adequate purity and that any lack of compliance with pharmacopoeial standard may be a result of manufacture. For this particular drug it appears that manufacture of the tablet and shampoo dosage form has been appropriate but that the content of the active ketoconazole is low in several (but not all) of the cream samples tested. A possible analytical cause of such low results could be inadequate extraction of active ingredient from the cream matrix. This is considered unlikely since the extraction and dilution procedure resulted in complete dispersal and dissolution of the cream so that filtration was not required. Also both external standard and standard addition methods when applied to the cream samples yielded the same results indicating the absence of matrix interference. Separate duplicate assay of cream samples confirmed the results obtained with only the same single cream sample complying with pharmacopoeial limits. In addition, a sample of ketoconazole cream obtained in the UK was analysed by the proposed method. This yielded a mean value of 98.7% of the stated amount (n = 5).

Conclusions

The method described for the assay of ketoconazole is capable of determining the content of this drug in the usual range of pharmaceutical formulations without interference from either excipients or the four specified related compounds. The day-to-day precision in terms of the constancy of the calibration slope is adequate as is the within-day precision even in the absence of an internal standard. It is also capable of detecting the recognised impurities associated with ketoconazole. The implications of the results obtained from the analysis of different dosage forms will be discussed more fully elsewhere in the context of ketoconazole and other drugs.

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