Electron Paramagnetic Resonance Method for the Quantitative Assay of Ketoconazole in Pharmaceutical Preparations

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In this study, electron paramagnetic resonance (EPR) is used, for the first time, as an analytical tool for the quantitative assay of ketoconazole (KTZ) in drug formulations. The drug was successfully characterized by the prominent signals by two radical species produced as a result of its oxidation with 400 µg/mL cerium(IV) in 0.10 mol dm⁻³ sulfuric acid. The EPR signal of the reaction mixture was measured in eight capillary tubes housed in a 4 mm EPR sample tube. The radical stability was investigated by obtaining multi-EPR scans of each KTZ sample solution at time intervals of 2.5 min of the reaction mixing time. The plot of the disappearance of the radical species shows that the disappearance is apparently of zero order. The zero-time intercept of the EPR signal amplitude, which should be proportional to the initial radical concentration, is linear in the sample concentration in the range between 100 and 400 µg/mL, with a correlation coefficient, r, of 0.999. The detection limit was determined to be 11.7 ± 2.5 µg/mL. The method newly adopted was fully validated following the United States Pharmacopeia (USP) monograph protocol in both the generic and the proprietary forms. The method is very accurate, such that we were able to measure the concentration at confidence levels of 99.9%. The method was also found to be suitable for the assay of KTZ in its tablet and cream pharmaceutical preparations, as no interferences were encountered from excipients of the proprietary drugs. High specificity, simplicity, and rapidity are the merits of the present method compared to the previously reported methods.

Ketoconazole (KTZ), cis-1-acetyl-4-[4-[[[2-(2, 4-dichlorophenyl)-2-(1H-imidazole-1ylmethyl)-1,3-dioxolan-4-yl] methoxy] phenyl] piperazine (Scheme 1) is a highly effective broad spectrum antifungal agent.¹⁻⁴ Recently, new drugs in which KTZ is coordinated to transition metals such as Ru, Rh, Cu, Au, and Pt result in a remarkable enhancement of the biological activity as antityrpanosoma cruzi.⁵⁻⁸ KTZ is also used to treat a wide variety of superficial and systemic mycoses and has the advantage over other imidazole derivatives of producing adequate sustained blood levels following oral administration.⁹

Several methods for the assay of KTZ have been reported, including potentiometric methods that are approved standard methods in the British Pharmacopoeia (BP) and United States Pharmacopoeia (USP).¹⁰,¹¹ Both methods are based on the potentiometric titration of the KTZ against 0.1 mol dm⁻³ perchloric acid in an anhydrous acetic acid media.

Intensive investigations were performed to assay KTZ using different spectrophotometric methods. Farhadi and Maleki¹² selected 1,10-orthophenanthroline (Phen) for indirect spectrophotometric determination of KTZ based on its oxidation with Fe(III) to produce a colored [Fe(Phen)]³⁺ complex which absorbs at 512 nm. Khashaba et al.¹³ suggested simple spectrophotometric and spectrofluorimetric methods for the determination of antifungal drugs such as clotrimazole, econazole nitrate, KTZ, miconazole, and tolnaftate. Similarly, Abdel-

Scheme 1. Ketoconazole (KTZ) Molecular Structure

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Gawd et al. established that KTZ reacts with iron(III) chloride in the presence of potassium thiocyanate to form a pink complex (2:1) that is soluble in 1,2-dichloroethane with a maximum absorbance at 510 nm.

Vojic et al. studied the acid–base equilibria of the diprotic, slightly hydrosoluble base KTZ in homogeneous and heterogeneous water systems at 25 °C at a constant ionic strength of 0.1 mol dm⁻³ NaCl solution. The acidity constants, $K_a$ and $K_{a2}$, served to calculate the solubility and the distribution of the equilibrium forms of KTZ as a function of pH. On the basis of the distribution of the equilibrium forms of KTZ and the absorbance at 225 nm, a spectrophotometric method for the determination of its content in commercial tablets was developed. Jalali et al. described a spectrofluorometric determination of KTZ from different complex formulations that has been applied to low concentration pharmaceutical preparations and blood serum samples, respectively. Kedor-Hackmann et al. developed a spectrophotometric method for quantitative determination of KTZ in commercial and simulated emulsion formulations using a zero-crossing method at 257 nm with methanol as the background solvent. About-Attia et al. developed a spectrophotometric method for the determination of three pharmaceutical piperazine derivatives, namely ketoconazole, trimetazidine hydrochloride, and piribedil, by the formation of yellow-orange complexes with iron(III) chloride. El-Ragehy et al. quantified KTZ by measuring complexes formed with copper(II) or cobalt(II) spectrophotometrically.

Arranz et al. described a capillary zone electrophoresis (CZE) method for simultaneous determination of a mixture of three imidazolic antifungal drugs. Abdel-Moety et al. developed a high-performance liquid chromatographic technique for the determination of clotrimazole, KTZ, and fluconazole, in pure forms and in pharmaceutical formulations.

Recently, electron paramagnetic resonance spectroscopy was successfully utilized in our laboratories as an analytical tool for the investigation of the free radicals generated in different systems including fermented natural leaves, degraded plastics, and irradiated alanine, and for the assay of chlorpromazine in drug formulations.

The present work is based on monitoring the radical systems formed as the result of oxidation of the KTZ with cerium(IV) in sulfuric acid media.

EXPERIMENTAL SECTION

KTZ Generic Samples. It is considered as the standard treatment for seborrheic dermatitis, being the active ingredient of Nizoral tablets and cream, as well as of an antidandruff shampoo. Due to its high permeability but low aqueous solubility, this drug is classified as a class II active substance, since its dissolution properties in the gastrointestinal tract are insufficient under normal conditions. Therefore, acidified double-distilled deionized water was used throughout for the preparation of the KTZ solutions and all other dilutions.

KTZ Proprietary Samples. Twenty tablets from proprietary drugs were accurately weighed, crushed, and powdered. The amount of the powder containing the appropriate weight to give 1000 µg/mL KTZ was dissolved in about 40 mL of acidified water, heated for 3 min, filtered, and then diluted to a 100 mL volume in a volumetric flask after being cooled. Further, samples were made by successive dilutions of this sample.

The cream sample solutions were prepared by dissolving a precise amount of the cream in hot 0.10 mol dm⁻³ sulfuric acid. The excipients were separated by filtration, and the filter paper was washed three times with the same acid. The filtrate and wash solutions of the cream samples were transferred quantitatively into a 100 mL volumetric flask and diluted to the mark with the same acid to give a 1000 µg/mL KTZ solution. Other diluted solutions are prepared from this stock.

Figure 1. EPR experimental (top) and simulated (bottom) spectra for the oxidation of 400 µg/mL KTZ in 0.1 mol dm⁻³ sulfuric acid with 400 µg/mL cerium(IV). Spectrometer settings are as follows: microwave frequency, 9.49397 GHz; microwave power, 5 mW; center field, 337 mT; sweep width, 10 mT; resolution, 4096 points; receiver gain, 2.5 × 10²; modulation frequency, 100 kHz; modulation amplitude, 0.1 G; time constant, 30.0 ms; sweep time, 120 s.

Figure 2. EPR signal using 5 (top) and 8 (bottom) capillary tubes housed in a 3 and 4 mm ESR sample tube, respectively, for the reaction mixture of 0.5 mmol dm⁻³ of KTZ and 50 µg/mL Ce(IV) after 2.5 min.
For the shampoo sample, an accurately weighed portion of the KTZ shampoo equivalent to 10 mg of the drug was dissolved in 0.10 mol dm$^{-3}$ sulfuric acid, filtered, and washed with hot acid. The supernatant liquid was made up to a volume of 100 mL in a volumetric flask and kept as the stock solution, from which other working solutions were prepared.

**Sulfuric Acid Solution (0.1 mol dm$^{-3}$).** A stock solution of 0.1 mol dm$^{-3}$ H$_2$SO$_4$ (98% specific gravity 1.84 kg dm$^{-3}$, Merck, U.K.) was prepared the usual way. Working solutions were prepared by dilution.

**Cerium(IV) Solution (1000 µg/mL).** A stock solution was prepared by dissolving a sufficient amount of dried cerium(IV) ammonium sulfate [Ce(NH$_4$)$_4$SO$_4$·2H$_2$O] (Fluka AG, CH-9470 Buchs, Switzerland) in 0.1 mol dm$^{-3}$ sulfuric acid and diluting to 1000 mL with the same acid solution in a calibrated flask.

**Electron Paramagnetic Resonance (EPR) Spectroscopy.** A JEOL ER-series EPR spectrometer operating at X-band frequency and a modulation frequency and amplitude of 100 kHz and 1 G, respectively, were used. The data were transferred to a PC and saved in a format so that they could be read by EXCEL, a program with which the data were analyzed. Microwave frequencies were measured with a Hewlett-Packard 5342 A microwave frequency counter.

### RESULTS AND DISCUSSION

Figure 1 shows two spectra: one of them for the radicals produced as a result of the oxidation of pure KTZ with cerium(IV) in 0.1 mol dm$^{-3}$ sulfuric acid and the second one presents its simulated spectrum. It is worth mentioning here that the simulated EPR spectra has been generated utilizing the WinSim EPR software from NIEHS.$^{31}$ The resultant simulated spectrum indicates that the drug undergoes fragmentation of the two ends of the KTZ compound producing imidazole and acetamide radical species. The spectral pattern of the former radical is well in agreement with a previously reported spectrum of a delocalized unpaired electron on an imidazole ring in a lophine-X anion radical.$^{32}$ The simulation also indicates that the imidazole and the acetamide radicals contribute to the total signal area by 83.3% and 16.7%, respectively.

Furthermore, the simulation indicated that the imidazole hyperfine splitting arises from two semiequivalent nitrogen atoms with hyperfine constants $a_N = 1.41 ± 0.02$ mT that will produce five distinct main groups with an intensity ratio of 1:4:6:4:1. These groups are due to three distinct sets of proton coupling, two on an imidazole ring and the third on an N-methylene group, with coupling constants $a_H = 0.28 ± 0.03$ (2 protons), $1.58 ± 0.02$ (1 proton), and $0.49 ± 0.03$ (2 protons) mT, respectively. On the other hand, the acetamide nitrogen produces three groups of $a_N = 0.97 ± 0.02$ mT; each of the group is due to two equivalent sets of protons around the amide functional group. A set consists of four protons with coupling constants equal to 0.43 ± 0.02 mT and the other one of two protons with a coupling constant equal to 0.21 ± 0.02 mT.

The lifetime of these radicals (about 15 min) is sufficient to allow one to search for the best EPR signal intensity using the univariant method.$^{33,34}$ The variables optimized were sulfuric acid concentration, sampling and sample size using capillary tubes, and cerium(IV) concentration. Testing a series of acid concentration in the range of 0.05–0.40 mol dm$^{-3}$, holding the concentration constant at 0.5 mmol dm$^{-3}$ KTZ, demonstrated that the optimal acid concentration is 0.1 mol dm$^{-3}$.

For the sampling and sample size, the study was limited to commercially available capillary tubes of 75 mm in length, 0.5–0.9 mm in internal diameter (i.d.), and 40 µL in full volume, because of the reported independency of signal intensity on the microwave power (MP) using a 100 µL flat cell.$^{35,36}$ Different numbers of tubes were housed in regular EPR sample tubes of different dimensions.

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The ratio of the radicals at 150 µg/mL found = (fixed time signal - standard fixed time signal)/slope of regression analysis. Percent recovery = amount found/amount spiked × 100.

Table 1. Recovery Tests of KTZ Solution Systems

<table>
<thead>
<tr>
<th>recovery level</th>
<th>amount spiked, mg/mL</th>
<th>fixed time signal</th>
<th>amount founda, mg/mL</th>
<th>% recoveryb</th>
<th>average recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (80%)</td>
<td>0.2020</td>
<td>1551</td>
<td>0.2018</td>
<td>99.89</td>
<td>100.6</td>
</tr>
<tr>
<td></td>
<td>0.2010</td>
<td>1545</td>
<td>0.2013</td>
<td>100.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1990</td>
<td>1558</td>
<td>0.2023</td>
<td>101.7</td>
<td></td>
</tr>
<tr>
<td>2 (90%)</td>
<td>0.2250</td>
<td>1923</td>
<td>0.2289</td>
<td>101.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2280</td>
<td>1944</td>
<td>0.2304</td>
<td>101.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2260</td>
<td>1951</td>
<td>0.2294</td>
<td>101.5</td>
<td></td>
</tr>
<tr>
<td>3 (100%)</td>
<td>0.2520</td>
<td>2218</td>
<td>0.2503</td>
<td>99.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2540</td>
<td>2215</td>
<td>0.2501</td>
<td>100.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2480</td>
<td>2209</td>
<td>0.2497</td>
<td>100.7</td>
<td></td>
</tr>
<tr>
<td>4 (110%)</td>
<td>0.2750</td>
<td>2556</td>
<td>0.2749</td>
<td>99.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2720</td>
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<td>0.2750</td>
<td>100.7</td>
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<tr>
<td></td>
<td>0.2760</td>
<td>2553</td>
<td>0.2747</td>
<td>99.54</td>
<td></td>
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<tr>
<td>5 (120%)</td>
<td>0.3010</td>
<td>2904</td>
<td>0.3003</td>
<td>99.76</td>
<td></td>
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<tr>
<td></td>
<td>0.3030</td>
<td>2885</td>
<td>0.2996</td>
<td>98.89</td>
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</tr>
<tr>
<td></td>
<td>0.3020</td>
<td>2901</td>
<td>0.3001</td>
<td>99.36</td>
<td></td>
</tr>
</tbody>
</table>

* Percent recovery = amount found/amount spiked × 100.

Table 2. Tablet and Cream Samples' Precision Test of KTZ Solution Systems

<table>
<thead>
<tr>
<th>trial # ATW, mg</th>
<th>amount spiked, g</th>
<th>fixed time signal</th>
<th>assay, mg</th>
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<tbody>
<tr>
<td></td>
<td>tablet cream</td>
<td>tablet cream</td>
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<td>1</td>
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<tr>
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<tr>
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<td>2375 1958</td>
<td>267.2</td>
</tr>
<tr>
<td>4</td>
<td>0.2530 0.2510</td>
<td>2376 1965</td>
<td>265.2</td>
</tr>
<tr>
<td>5</td>
<td>0.2500 0.2520</td>
<td>2377 1958</td>
<td>268.5</td>
</tr>
<tr>
<td>6</td>
<td>0.2540 0.2520</td>
<td>2373 1958</td>
<td>263.8</td>
</tr>
</tbody>
</table>

EPR Method Validation. The EPR method of KTZ drug formulation is validated following the USP monograph protocol. The linearity was demonstrated for the standard solution over a range of 50–150% of a solution containing 250 µg/mL KTZ using 400 µg/mL cerium(IV) and 0.1 mol dm⁻³ sulfuric acid. The zero-time intercept of the EPR signal amplitude, which should be proportional to the initial radical concentration, is linear in the KTZ concentration range between 100 and 200 µg/mL while the richer reaction system of imidazole radicals was linear over much wider range of KTZ, 150 to 400 µg/mL.

Analytical Appraisals. Under the optimal conditions investigated above, a series of solutions of different KTZ concentrations was scanned at different fixed times between 2.5 and 12.5 min with time intervals of 2.5 min using 200 µg/mL or 400 µg/mL Ce(IV) solutions. Regardless the type of radical species, the plot of the disappearance of the radical species shows that the disappearance is apparently of zero order. Moreover, the zero-time intercept of the EPR signal amplitude, which should be proportional to the initial radical concentration, is linear in the sample concentration. The reaction system with a larger quantity of acetamide radicals was linear in the KTZ concentration range between 100 and 200 µg/mL while the richer reaction system of imidazole radicals was linear over much wider range of KTZ, 150 to 400 µg/mL.
where I is the intercept of the linear plot of the radical decay and C is concentration in micrograms per milliliter. The detection limit was determined to be 11.7 ± 2.5 µg/mL using the equation: DL = (3.3 × s/S ± t_{p,v} σ/s), where DL is the detection limit, s is the standard deviation of responses, S is the slope of the calibration curve, and the uncertainty in DL is amplified by the confidence interval for σ/s. The large intercept value is certainly due to the formation of an irreversible fragmentation reaction once the reaction mixture takes place. This phenomenon has been observed by the formation of an instantaneous brown color upon mixing, which slowly decays with time.

The recovery tests were performed against the standard solution by preparing the drug without excipients at an 80, 90, 100, 110, and 120% level of the final sample concentration. The results are listed in the Table 1 and indicate that the recovery test of the EPR assay approaches 100% (average recovery = 100.3 ± 0.8%).

The precision of the EPR assay procedure was also investigated by the determination of the standard deviation (SD) of the repeatability of the method for six determinations of tablet and cream samples under the same analytical conditions. The listed results in Table 2 indicated that the EPR is also highly reproducible as the precision of the results obtained for the tested tablet and cream samples were found to be quite reasonable, %SD = 0.76 and 1.32%. In shampoo samples, no radical was detected, most probably due to the quenching ability of the shampoo excipients.

The accuracy of the method was investigated by assaying the same batch of proprietary drugs of KTZ using the EPR method and the standard USP method. The results obtained are chemometrically compared by calculating the student t test values introduced in Table 3. All calculated t test values were found to be less than the tabulated values at the 99.9% confidence level, indicating accuracy of the EPR method. In addition, the results obtained also indicate that the present method suffers no interferences from the excipients already existing in these formulations.

**CONCLUSIONS**

The EPR spectroscopic technique has proven to be successful for the quantitative determination of KTZ in tablet and cream preparations. The method thus adopted was found to be precise, accurate, specific, and fast and consumed less reagents compared to the previously reported methods.

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