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Evaluation of a CGC–FID Method for Qualitative and Quantitative Analysis of Azole Antifungal Drugs

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Summary. This paper presents a new method for identification and quantitative analysis of six azole antifungal drugs – bifonazole, clotrimazole, econazole, fluconazole, ketoconazole, and miconazole – by capillary gas chromatography (CGC) combined with flame ionization detection (FID). The chromatographic separation conditions were established and the method was validated for precision (RSD = 1.49–3.55%), recovery (98.6–101.2%), and linearity within the range under investigation (~1.0–33.3 ng). The results obtained show the newly developed procedure is suitable for qualitative and quantitative pharmaceutical analysis of the six azoles.

Key Words: azole antifungal drugs, pharmaceutical analysis, capillary gas chromatography, flame-ionization detection

Introduction

Among the synthetic antifungal substances used in therapy, the azole compounds imidiazole and triazole derivatives are the most important. Because of high efficacy and an acceptable safety profile these compounds have gained a strong position in therapy [1–3].

These compounds have been analysed by use of a variety of techniques, including spectrophotometry [4] and biological [5] and electroanalytical [6] methods. Chromatographic methods, for example high-performance liquid chromatography (HPLC) [7] and thin-layer chromatography [8] have also been used. There are, however, a few publications describing gas chromatographic analysis. Bifonazole and econazole have been analysed by GC–FID [9], clotrimazole by CGC with mass spectrometric de-

tection (MSD) [10], ketoconazole by CGC with electron-capture detection (ECD) [11], and miconazole by GC with packed columns [12, 13]. Fluconazole, a triazole derivative, has been analysed by CGC with a variety of detection methods – flame ionization detection (FID) [14], mass spectrometry (MS) [14, 15], nitrogen–phosphorus detection (NPD) [16, 17], and ECD [18, 19]. This paper reports capillary gas chromatographic analysis of the imidazole derivatives bifonazole, clotrimazole, econazole, ketoconazole, and miconazole, and of the representative triazole derivative fluconazole.

FID, although the most common universal mode of detection in gas chromatography, has not been explored sufficiently in analysis of these compounds. The objective of our work was to develop and validate a CGC–FID method for identification and determination of these six antifungal drugs.

Experimental

Reference Standards, Reagents, Preparations

Analysis was performed on methanol solutions of all the azole compounds. The internal standard (IS), cholesterol, was also dissolved in methanol. All the standards were supplied by Sigma (St Louis, USA) and met pharmacopoeial requirements. Methanol of analytical quality was from POCh (Gliwice, Poland).

Ketoconazole solution at a concentration of 1 mg mL^{-1} and 0.05 mg mL^{-1} internal standard, and solutions of concentration 1 mg mL^{-1} analyte and 0.5 mg mL^{-1} internal standards for the other azole compounds were used for analysis (standard solutions). The different concentrations of the internal standard resulted from adjustment of the height of its peaks to those of the analytes. Two of the substances investigated, econazole and miconazole, occurred as the nitrate salts. Solutions were kept at low temperature (4°C) and were protected from light.

The analytical conditions established for reference standards were used for quantitative analysis of selected analytes in medicinal products. The studies were conducted on the preparation Ketoconazole, 200 mg tablets (Anpharm) and on the drug Fluconazole, 50 mg tablets (Polfarmex). The tablets were powdered, and an amount of powder equivalent to the average weight of a tablet was mixed with methanol and shaken for 15 min at a frequency of approximately 3 cycles s^{-1}. The solution was then filtered through no. 2 filter paper. The prepared solutions were of concentration 2 mg mL^{-1}. Sample solutions were obtained by mixing of an appropriate amount of the prepared solution with internal standard solution in methanol (2 mg mL^{-1})

and diluting with methanol to the required volume. The concentration of the active ingredient was 1 mg mL^{-1} for both ketoconazole and fluconazole samples, and the internal standard concentrations were 0.05 and 0.5 mg mL^{-1}, respectively.

Chromatography

Gas chromatography was performed with a Trace GC, with flame ionization detection (FID), controlled by a PC in which Chrom Card for Trace software was installed (Thermo Finnigan, Rodano, Italy),. Compounds were separated on a 15 m × 0.25 mm i.d. wall-coated open tubular (WCOT) HP-1 column (Hewlett–Packard, Palo Alto, USA). The column was coated with a 0.1 μm film of the non-polar polydimethylsiloxane stationary phase OV-1.

Manual split injection (split ratio 1:30) of approximately 1-μL samples was performed at an inlet temperature of 300°C. The detector temperature 330°C. After injection the oven temperature was increased quickly (40° min^{-1}) from 50°C, which was held for 1 min, then programmed within 7 min to 330°C which was held for 4.5 min. The final isothermal period was reduced to 2 min, if possible (i.e. if no further peaks were eluting) during replicate injections.

Helium at a flow rate of 1 mL min^{-1} was used as carrier gas. Synthetic air (flow rate 350 mL min^{-1}), hydrogen (35 mL min^{-1}), and nitrogen as make-up gas (34 mL min^{-1}) were fed to the FID. All the gases used in these studies were of N5.0 purity from Linde Gaz Polska (Kraków, Poland).

Azole compounds and cholesterol were analysed without prior derivatization. Analysis was performed directly and the internal standard technique was used for computation.

Results

Qualitative Analysis

Individual chromatograms were recorded for all the azole compounds with and without internal standard. Examples of chromatograms obtained from ketoconazole, as imidazole representative, and fluconazole, as triazole, are shown in *Figs 1* and *2*, respectively. Both chromatograms show the peak of cholesterol, used as internal standard.

On the basis of the chromatograms obtained, characteristic retention times relative to that of cholesterol were determined for the drugs as the basis for qualitative identification (*Table I*).

Table I. Relative retention times (*RRT*) of the azole compounds

Compound	Relative retention time (RRT)
Bifonazole	0.94
Clotrimazole	0.90
Econazole nitrate	0.92
Fluconazole	0.72
Ketoconazole	1.56
Miconazole nitrate	0.96

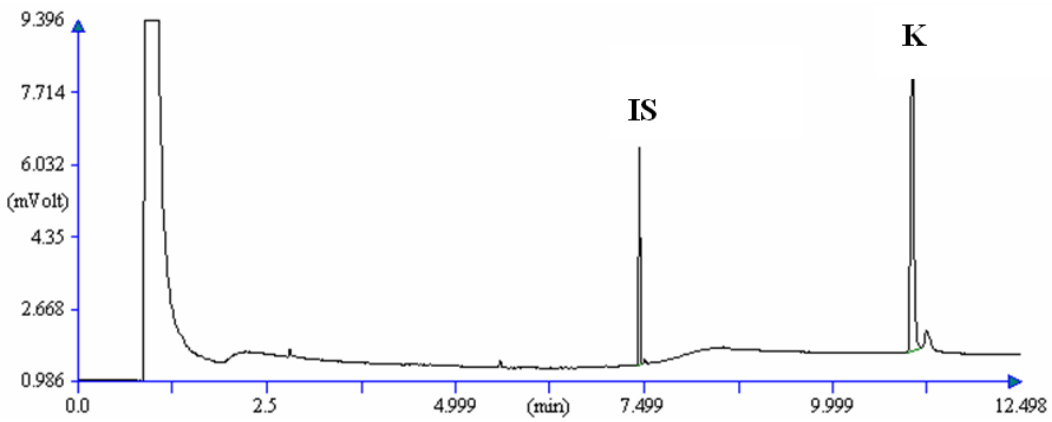

Fig. 1. Chromatogram obtained from ketoconazole (K) and cholesterol (IS) solution

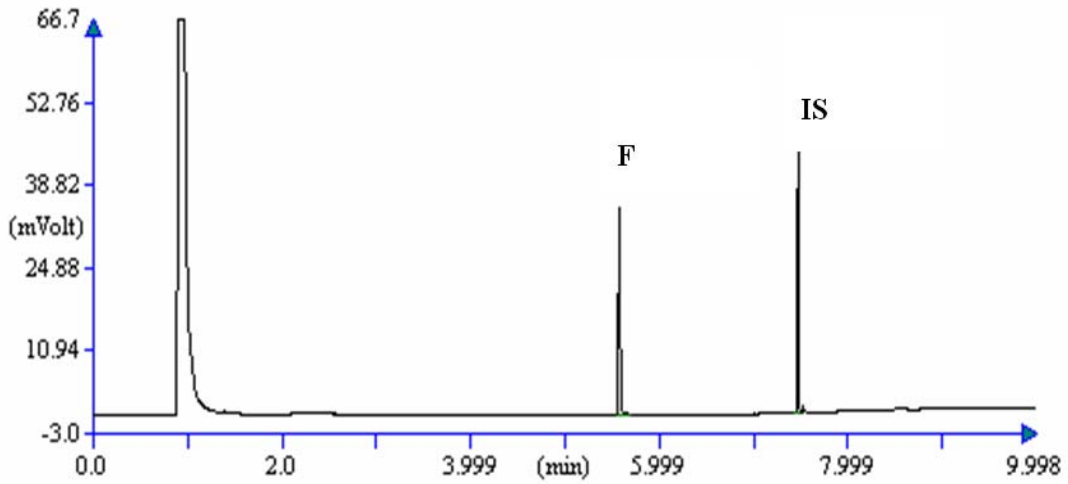

Fig. 2. Chromatogram obtained from fluconazole (F) and cholesterol (IS) solution

The range of relative retention times for the individual azoles was considered useful for their simultaneous identification and quantitative analysis. For this purpose a mixture of each of the analysed compounds at a concentration of 1 mg mL^{-1} and the internal standard at 0.5 mg mL^{-1} was prepared. The chromatogram obtained from this solution is shown in *Fig. 3*.

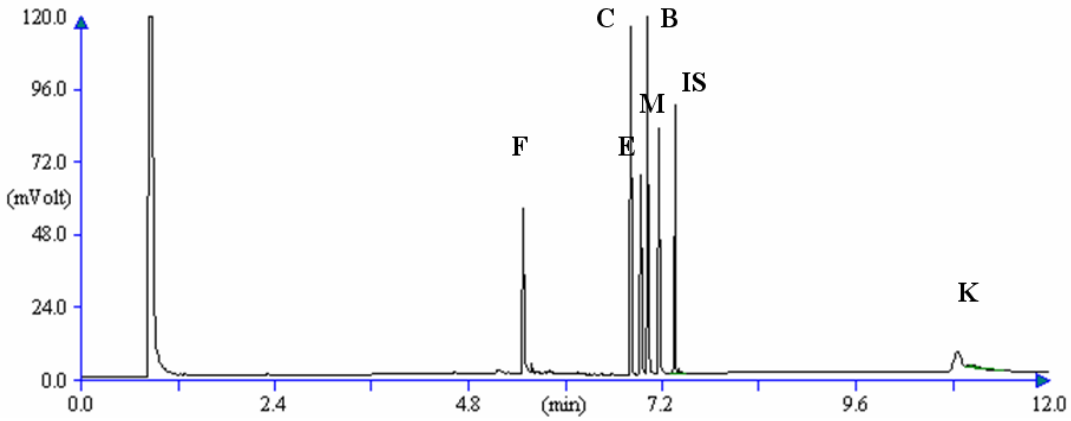

Fig. 3. Chromatogram obtained from a mixed solution containing fluconazole (F), clotrimazole (C), econazole nitrate (E), bifonazole (B), miconazole nitrate (M), cholesterol (IS), and ketoconazole (K)

Peaks within retention time (t_R) range of 6.6–7.5 min did not differ much in value but the peaks were completely separated ($R_S > 0.5$) and could be the basis for analysis (*Fig. 4*). The choice of cholesterol as internal standard was justified by its closeness on the chromatogram to almost all the drugs investigated and its sufficient resolution ($R_S > 1.25$).

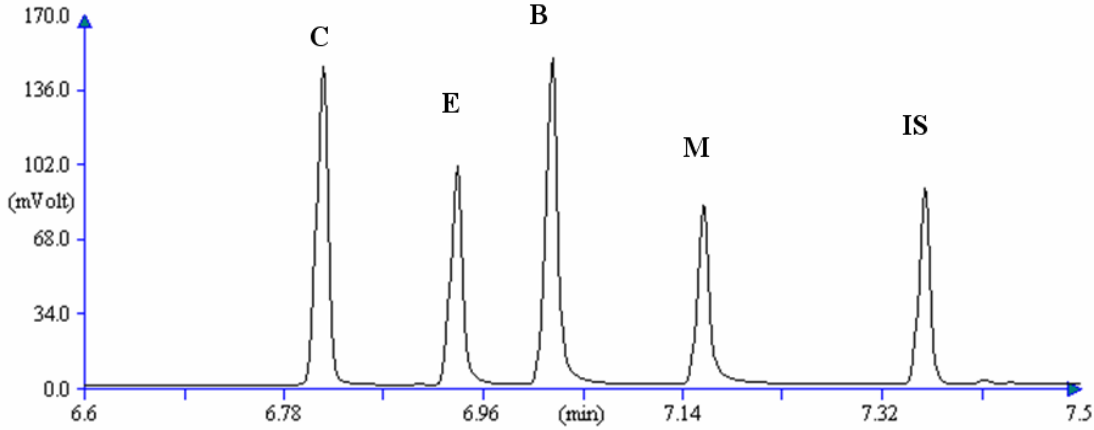

Fig. 4. Chromatogram obtained from a mixed solution, focussing on the range 6.6–7.5 min. C, clotrimazole; E, econazole nitrate; B, bifonazole; M, miconazole nitrate; IS, cholesterol

Method Validation and Quantitative Analysis

After the method conditions were established – as described above – the method was validated for precision, accuracy, and linearity within the specified range of amounts [20]. Precision was measured as the repeatability of a series of results (n = 6) and was also checked inter-day. Accuracy was determined as percentage recovery (n = 3) at three concentrations (80, 100, and 120% of the amount expected) achieved by spiking placebo with reference standards. Linearity was established by chromatography of a series of solutions (n = 5) of decreasing concentration. The limits of detection (*LOD*) and quantification (*LOQ*) were defined as the amounts for which the ratio of the signal (S) to the baseline noise (N) were 1:3 and 1:10, respectively.

Quantitative analysis of the drugs was performed under the conditions established. Ketoconazole and fluconazole were analysed in medicinal products in the form of immediate release tablets (*Table II*). For these compounds method selectivity was evaluated by comparing t_R values in chromatograms obtained from the analysed product with those in chromatograms obtained from reference standards. No interference from auxiliary substances (i.e. no additional peaks) was observed. The ketoconazole preparation contained lactose, potato starch, microcrystalline cellulose, polyvidon, talc, and magnesium stearate as auxiliary substances whereas the fluconazole product contained lactose, maize starch, starch glyconate sodium salt, polyvidon, and magnesium stearate. Recovery of 80, 100, and 120% reference standard added to the placebo was 101.2, 98.6, and 100.8%, respectively, for ketoconazole and 100.7, 99.4, and 101.0%, respectively, for fluconazole.

Table II. Results from analysis of ketoconazole and fluconazole in medicinal products

	Ketoconazole tablets, 200 mg	Fluconazole tablets, 50 mg
Mean (mg)	201.1	50.7
Confidence interval at 95% (mg)	197.1–205.2	49.8–51.5
Precision (RSD, %)	2.02	2.37
Recovery (%)	98.6–101.2	99.4–101.0
Range (ng)	1.5–33.3	1.1–33.3
Linearity (r)	0.99856	0.99899
LOD (ng)	0.5	0.4
LOQ (ng)	1.5	1.2

A chromatogram obtained from placebo containing all the excipients and imidazole is shown in *Fig. 5*. Imidazole is a potential impurity of this class of drugs. The chromatogram was recorded after injection split 10 with an imidazole concentration of 1 mg mL^{-1}. Only the imidazole peak (t_R = 2.342 min) can be seen on the chromatogram. Neither imidazole nor placebo constituents affected the analytical results.

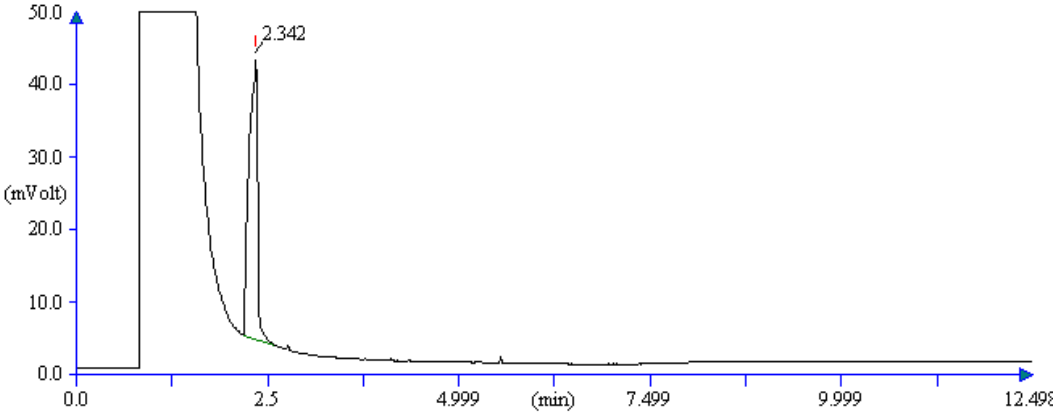

Fig. 5. Chromatogram obtained from placebo and imidazole (t_R = 2.342 min) solution

Results obtained from validation of the method for bifonazole and clotrimazole are listed in *Table III*; those obtained for econazole nitrate and miconazole nitrate are shown in *Table IV*.

Table III. Validation data for analysis of bifonazole and clotrimazole

	Bifonazole	Clotrimazole
Mean (0.1 μV s)	1 277 395	2 111 107
95% confidence interval (0.1 μV s)	1 257 355–1 297 435	2 069 310–2 152 904
Precision (RSD, %)	1.49	2.47
Range (ng)	0.7–33.3	2.2–33.3
Linearity (*r*)	0.99810	0.99735
LOD (ng)	0.2	0.7
LOQ (ng)	0.7	2.1

Table IV. Validation data for analysis of econazole nitrate and miconazole nitrate

	Econazole nitrate	Miconazole nitrate
Mean (0.1 μV s)	1 564 927	1 526 685
95% confidence interval (0.1 μV s)	1 520 458–1 609 396	1 498 317–1 555 053
Precision (RSD, %)	3.55	2.32
Range (ng)	1.4–33.3	0.4–33.3
Linearity (*r*)	0.99659	0.99121
LOD (ng)	0.5	0.1
LOQ (ng)	1.4	0.3

These validation data indicate the method is suitable for analysis of these compounds.

Discussion

It should be noted that reference monographs for qualitative analysis of all the compounds under examination, included in European Pharmacopoeia [21], require analysis of reference standards by titration with potentiometric determination of the end-point. This method is, however, unsuitable for analysis these compounds in medicinal products or biological material, where the presence of several other substances can affect the analytical results. For analysis of such complex samples as drugs, a selective method with high separation potential is required. Gas chromatography meets these criteria.

The United States Pharmacopeia [22] contains monographs not only for azoles as reference substances but also for specific formulations containing these compounds. For the substances alone titrimetry is usually recommended (liquid chromatography for clotrimazole). Analysis of preparations depends on their character and recommended methods include liquid and gas chromatography. Gas chromatography with FID is used for analysis of the active substance in miconazole nitrate topical powder and miconazole nitrate vaginal suppositories. Recommended conditions differ from those presented in this paper – a packed column and isothermal column temperature are used. It is well known that the performance of capillary columns is much better than that of packed columns, so this should be treated as advantage of the method reported in this publication.

Various detection techniques can be used in gas chromatography. For the compounds under consideration it is justified to use ECD (except for bifonazole, which contains no electronegative halogens), NPD, or MS. The FID is, however, the universal GC detector. So far, only bifonazole with

econazole [9] and fluconazole [14] have been analysed by use of FID. For analysis of bifonazole a packed column was used and prenylamine lactate served as the internal standard. The method proposed in this paper involves use of a capillary column with much better performance.

In the second publication [14] fluconazole was analysed by CGC–FID with propiophenone as internal standard. On-column injection was performed and a temperature programme in the range 45–280°C was used. Dichloromethane was used as a solvent. Complete information about method validation is not given, no chromatograms are presented, and no analyte and internal standard retention times are reported. The objective of the work reported in that paper was not to present complete data related to analysis, especially qualitative analysis, which makes it difficult to compare the analytical procedures. The objective of that method was to enable determination of fluconazole in animal feed.

The method proposed in this paper enables analysis of six chemically closely related antifungal drugs using the same chromatographic conditions and the same internal standard.

Conclusion

This paper presents the first method for identification and separation of the six common azole compounds by use of capillary gas chromatography with flame ionization detection (CGC–FID). This procedure for quantitative analysis of bifonazole, clotrimazole, econazole, ketoconazole, miconazole, and fluconazole is suitable for routine analysis of these compounds in medicinal products. Previously, CGC–FID methods have been developed for fluconazole analysis only, so this is the first such procedure for all the other drugs under investigation. The method can probably also be applied to analysis of these compounds in biological and environmental materials after appropriate preliminary preparation.

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