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Gas chromatographic–mass spectrometric determination of two new antimycotic agents, 1-[(5-chloro-2-benzofuranyl)(2-chlorophenyl)methyl]-1*H*-imidazole and 1-[(5-bromo-2-benzofuranyl)phenylmethyl]-1*H*-imidazole, in rabbit plasma following topical administration: a preliminary comparison with bifonazole

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ABSTRACT

A method for the analysis of the antimycotic drugs 1-[(5-chloro-2-benzofuranyl)(2-chlorophenyl)methyl]-1*H*-imidazole, 1-[(5-bromo-2-benzofuranyl)phenylmethyl]-1*H*-imidazole and bifonazole in rabbit plasma, employing gas chromatography–mass spectrometry with selected-ion monitoring, was developed. The procedure involved single-step purification of the biological matrix via liquid–liquid extraction on Extrelut™ columns and use of a carrier substance to minimize the negative effects of adsorption sites during the gas chromatographic process. The limits of detection ranged from 0.1 to 1.4 ng/ml, starting from a 200-μl sample. The method was applied to a preliminary evaluation of percutaneous absorption of both drugs in the rabbit after a single administration, in comparison with bifonazole.

INTRODUCTION

1-[(5-Chloro-2-benzofuranyl)(2-chlorophenyl)methyl]-1*H*-imidazole (I, CAS 112893-26-2) and 1-[(5-bromo-2-benzofuranyl)phenylmethyl]-1*H*-imidazole (II, CAS 111790-32-0) (Fig. 1A and B) are two new antimycotic drugs belonging to the imidazole class [1-3]. It has been demonstrated that antimycotics of this family exert their activity by altering the fungal plasma membrane via inhibition of the biosynthesis of ergosterol, which plays an essential role in stabilizing membrane phospholipids [4]. From a biochemical point of view, this inhibition is due to the interaction of the azole with a cytochrome P-450-depending enzyme complex, which is involved in oxidative demethylation of a precursor of ergosterol, 24-methylenedihydrolanosterol. According Berg *et al.* [4], this can account for the side-effects of azole antimycotics, which are generated essentially by interaction with liver cytochrome P-450s involved in the metabolism of xenobiotic and endo-

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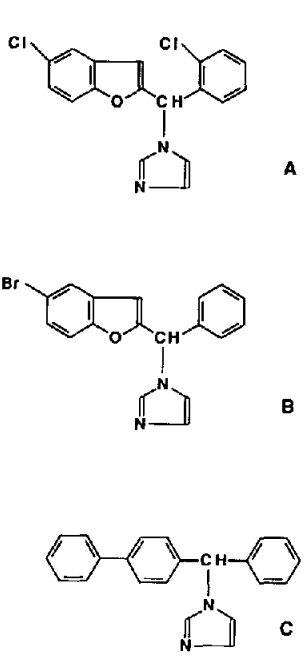

Fig. 1. Structures of I (A), II (B) and bifonazole (C).

genous substances, and those involved in oestrogen biosynthesis. Owing to these interactions, only a few azole drugs, *i.e.*, those showing a high degree of selectivity towards fungal cytochrome P-450, can be employed in the systemic treatment of mycoses. The other drugs can be administered topically, provided they are adsorbed in very low amounts from their application site.

The aim of this work was to make a preliminary evaluation of the degree of percutaneous absorption of I and II following a single topical application of 2 g of cream (2%) in the rabbit. For comparison, bifonazole, 1-[4-(1,1′-biphenylyl)-phenylmethyl]-1*H*-imidazole (Fig. 1C), was tested because it is known to be absorbed only to a very limited extent when given topically (less than 1% of the administered dose in humans) [5]; this drug was administered at a dose of 2 g of cream (1%).

Given the very low plasma levels expected for all three drugs, a very sensitive method was needed for their determination. Previous papers described a bifonazole assay in biological matrices using the radioactively labelled compound [6], high-performance thin-layer chromatography (HPTLC) with a limit of detection of 1 ng/ml [5,7–9] and high-performance liquid chromatography (HPLC) [5] with a limit of detection of 10 ng/ml.

This paper describes a gas chromatographic–mass spectrometric (GC–MS) method with selected-ion monitoring (SIM), which is capable of determining I, II and bifonazole in rabbit plasma in very low concentrations, starting from very small amounts of biological sample (200 μl).

EXPERIMENTAL

Materials

I and II were synthesized at Chemistry Department, A. Menarini (Florence, Italy). Bifonazole was purchased from Sigma (St. Louis, MO, U.S.A.). Extrelut 1™ columns were obtained from Merck (Darmstadt, Germany). *n*-Hexane was obtained from Baker (Phillipsburg, NJ, U.S.A.) and was "Baker Capillary Analyzed" grade. All the other solvents were analytical grade and were obtained from Carlo Erba (Milan, Italy) and Merck. For sample incubation and evaporation a Reacti-Therm™ module and a Reacti-Vap™ evaporating unit (Pierce, Rockford, IL, U.S.A.) were used.

Methods

For the analysis of each of the three antimycotic drugs, the internal standard and the carrier substance were chosen from the two other drugs, according to the scheme shown in Table I.

TABLE I

INTERNAL STANDARDS AND CARRIER SUBSTANCES USED FOR THE ANALYSIS OF EACH ANTIMYCOTIC DRUG

Analyte	Internal standard	Carrier
I	II	Bifonazole
II	I	Bifonazole
Bifonazole	II	I

In each case, the sample work-up procedure was as follows: 200 μl of sample were pipetted in a siliconized test-tube, then 20 ng of internal standard and 5 μg of carrier substance were added. The sample was vortex-mixed briefly and kept for 30 min at 37°C; 200 μl of 0.01 *M* NaOH were then added, and the sample was quantitatively transferred to an Extrelut 1 column, previously washed with 20 ml of chloroform and dried for 15 min under vacuum. After equilibration for 5 min, the sample was eluted with two 3-ml volumes of chloroform–*n*-hexane (1:1) and collected in a siliconized conical test-tube. It was then evaporated under a stream of nitrogen and dissolved in 20 μl of benzene–2-propanol (9:1) for GC–MS analysis.

Standards were prepared by adding various amounts of the analyte in the range 0.5–20 ng to 200-μl aliquots of blank rabbit plasma, together with 20 ng of internal standard and 5 μg of carrier. Each standard was subjected to the same extraction and purification procedure as the sample.

Gas chromatography–mass spectrometry

GC–MS was performed on an HP-5890 gas chromatograph coupled to an HP-5988A mass spectrometer (Hewlett-Packard, Avondale, PA, U.S.A.). For the chromatographic separation an Ultra-1 (100% methylsiliconc) fused-silica capillary column (25 m × 0.32 mm I.D., 0.52 mm film thickness, Hewlett-Packard) was used, directly connected to the ion source. The oven temperature was programmed from 85°C (0.5-min hold) to 220°C at 45°C/min and at 30°C/min up to 300°C. The sample injection volume was 1 μl. Helium was used as the carrier gas at an inlet pressure of 100 kPa.

The ion-source and transfer-line temperatures were set at 200 and 310°C, respectively. Both full-scan mass spectra and SIM experiments were performed in the electron-impact (EI) mode at an ionizing energy of 70 eV. Selected ions monitored were *m/z* 275 for I, *m/z* 287 for II and *m/z* 243 for bifonazole. For quantitation, the chromatographic peak-area ratios of known amounts of each analyte and its internal standard were plotted against the corresponding concentration ratios, and the resulting linear regression equation was calculated. Unknown samples were quantitated according to this equation.

RESULTS AND DISCUSSION

Full-scan spectra of I, II and bifonazole are illustrated in Fig. 2. They all show good suitability for SIM analysis, since in each case the base peak is an abundant ion generated from the molecular ion by loss of an imidazole radical, *m/z* 275, *m/z* 287 and *m/z* 243, respectively. For I and II, monitoring two or more peaks of the isotopic pattern of the base peak, generated by the occurrence of chlorine and

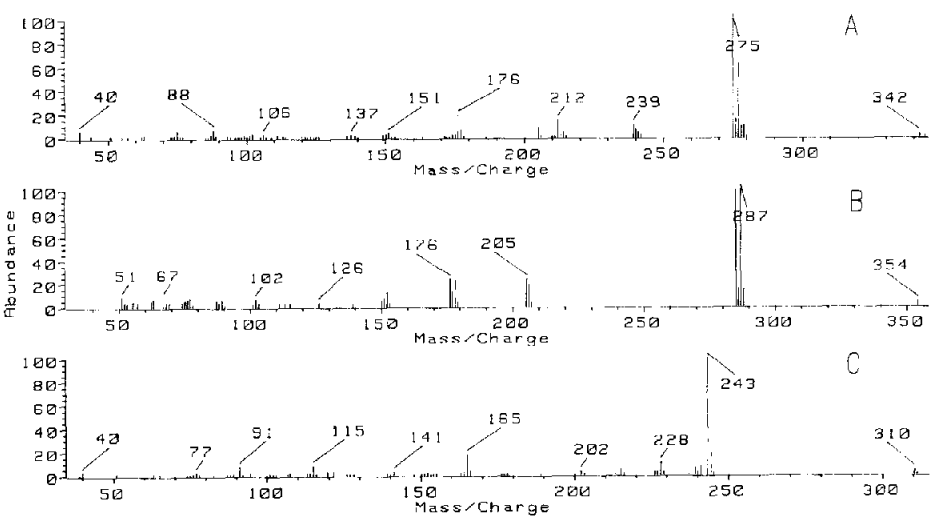

Fig. 2. Mass spectra of I (A), II (B) and bifonazole (C).

bromine atoms in the molecule, is potentially a useful means to increase specificity of the assay. However, we chose to monitor only the highest peak of the pattern, owing to the occurrence of more interferences in the selected-ion chromatograms of the other isotopical peaks.

A problem to overcome in GC–MS analysis of these molecules was their poor GC properties. Various stationary phases and film thicknesses were tested: the best results were obtained with 100% cross-linked methylsilicone and a thickness of 0.52 μm. In spite of this the chromatographic peaks of the compounds of interest were strongly tailed, and tailing increased very rapidly after few injections of biological sample, even when the shapes of the other peaks in the chromatogram were not worsening. Since there was no simple derivatization available for these molecules, a carrier substance was used to saturate the adorption sites in the column; in this way a significant improvement in peak shape was achieved, with up to three times baseline peak-width reduction.

Since generally a carrier substance has a better effect the closer its retention time is to the retention time of the analyte, and the greater its molecular similarity is to it, bifonazole was chosen as carrier for both I and II, and I was chosen for bifonazole analysis. In no case did the chromatographic peak of the carrier interfere with those of the analytes or internal standard; in particular, I coelutes with II, the internal standard for bifonazole analysis, but there is no appreciable contribution to the internal standard peak at the carrier concentration used. Typical selected-ion chromatograms are shown in Figs. 3–5.

For sample preparation, both solvent and Extrelut column extraction of the drugs were attempted. Solvent extraction with *n*-hexane under alkaline condi-

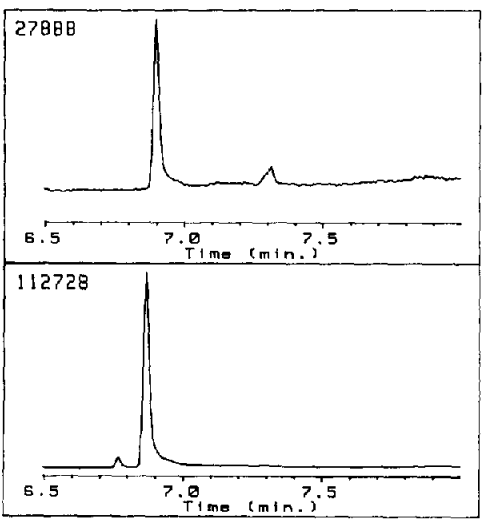

Fig. 3. Typical selected-ion chromatogram from rabbit plasma 2 h after topical administration of I (2 g of cream, 2%). Upper trace: ion *m/z* 275.0; lower trace, ion *m/z* 287.0 (internal standard).

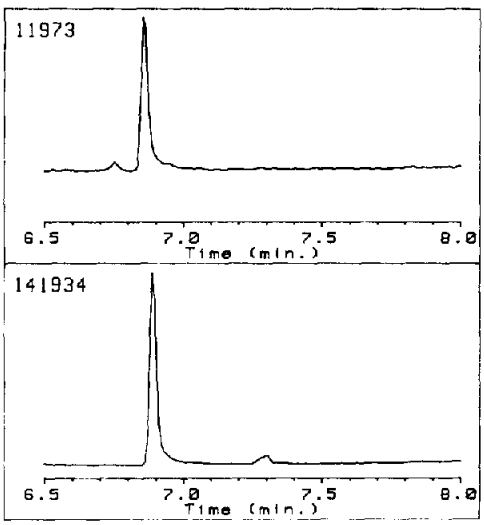

Fig. 4. Typical selected-ion chromatogram from rabbit plasma 2 h after topical administraton of II (2 g of cream, 2%). Upper trace, ion *m/z* 287.0; lower trace, ion *m/z* 275.0 (internal standard).

tions gave good recoveries, but required further purification owing to the high background. This was achieved by passing the extract through a silica mini-column, but this obviously resulted in an increased analysis time. Sample purification via liquid–liquid extraction with Extrelut columns, on the other hand,

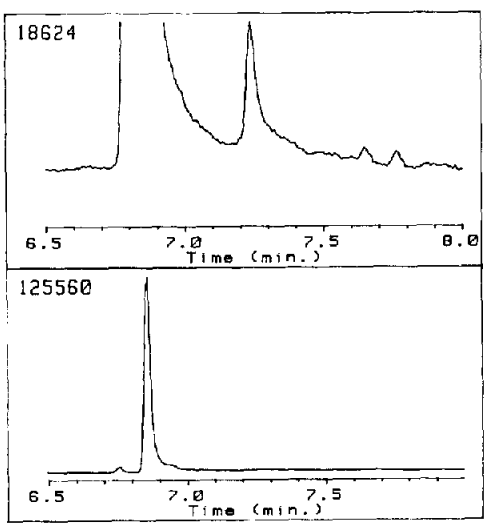

Fig. 5. Typical selected-ion chromatogram from rabbit plasma 2 h after topical administration of bifonazole (2 g of cream, 1%). Upper trace, ion *m/z* 243.1; lower trace, ion *m/z* 287.0 (internal standard). The peak off the scale in the upper trace is due to the carrier substance.

TABLE II

TYPICAL PARAMETERS FOR THE REGRESSION LINE EQUATIONS OF I, II AND BIFONAZOLE

Compound	Slope	Intercept	r^2	Number of points
I	1.39	0.0972	0.998	5
II	0.609	0.00962	0.999	5
Bifonazole	2.28	0.0118	0.997	5

yielded satisfactory isolation of all three drugs from plasma in one step, and a clean baseline.

The calibration curves showed a linear response in the range 2.5–100 ng/ml for all the analytes; typical parameters for the regression line equations are listed in Table II.

The limits of detection were estimated from the calibration curves by calculating the upper 95% confidence limits of the intercepts: they were 1.4, 0.4 and 0.1 ng/ml, respectively for I, II and bifonazole.

The intra-day precision and accuracy of the method were investigated by assaying rabbit plasma samples spiked with various amounts of the analytes; (Table III). The accuracy was in the range 93.6–102.6%. The coefficients of variation ranged from 6.7 to 7.5%, excluding the lowest concentration of I, which gave an appreciably higher value (18.2%).

The method was applied to the determination of I, II and bifonazole in the plasma of rabbits after a single topical application of 2 g of cream. The drug percentages in the cream were 2% for I and II, and 1% for bifonazole. Blood samples were collected 2 and 24 h after administration and frozen until analysis: the results are shown in Table IV.

Although a correct interpretation of these data requires knowledge, not yet

TABLE III

ACCURACY AND INTRA-DAY PRECISION DATA IN RABBIT PLASMA

In all cases, $n = 5$.

Compound	Concentration given (ng/ml)	Concentration found (ng/ml)	Accuracy (%)	R.S.D. (%)
I	19.3	19.8	102.6	6.7
	9.6	9.6	100.0	18.2
II	26.3	26.6	101.1	6.8
	8.8	8.6	97.7	7.5
Bifonazole	10.9	10.2	93.6	7.1
	5.5	5.2	94.5	7.4

TABLE IV

ANTIMYCOTIC DRUGS CONCENTRATION FOUND IN RABBIT PLASMA AFTER TOPICAL ADMINISTRATION

The doses were 2 g of cream (2%) for I and II, and 2 g of cream (1%) for bifonazole.

Time (h)	Concentration (mean ± S.E., $n = 5$) (ng/ml)		
	I	II	Bifonazole
2	15.3 ± 2.6	11.1 ± 0.6	4.2 ± 0.9
24	13.7 ± 3.4	7.8 ± 2.4	6.8 ± 1.2

available, of pharmacokinetic behaviour of the drugs, they indicate that percutaneous absorption of the antimycotic drugs I and II is of the same order of magnitude as bifonazole.

CONCLUSION

The method described here features a very simple sample work-up, a good specificity and a high sensitivity, which allows dosage of very low drug concentrations. Thus it is suitable for percutaneous absorption studies, which involve plasma concentrations in the low-nanogram range.

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