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Note

Gas chromatographic method for the determination of fluconazole, a novel antifungal agent, in human plasma and urine

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Fluconazole (UK-49,858) (Fig. 1) is a novel, potent, broad-spectrum triazolyl antifungal agent developed in our laboratories for oral and intravenous use in systemic and superficial infections in man [1]. The drug is excreted predominantly unchanged in the urine and there are no bioactive metabolites [2]. A feature of the compound is its potent in vivo activity; typical dose regimens in clinical therapy of fungal infections are 50 mg daily for 3 days (vaginal candidosis) or 28 days (systemic mycoses and dermatomycoses), with corresponding plasma concentrations being less than 2 μg/ml. However, in common with several other azole antifungals in clinical use [3], measurement of in vitro activity of the compound is unreliable, especially on solid media. Consequently bioassay is limited to concentrations in plasma which are higher than 1 μg/ml. A specific and sensitive chemical assay method was therefore required to determine concentrations of the drug in biological fluids prior to an assessment of the pharmacokinetics and bioavailability in animals and man.

Preliminary studies using gas chromatography (GC) showed that the drug was strongly adsorbed on columns containing conventional packing materials, resulting in poor peak shape and inadequate separation.

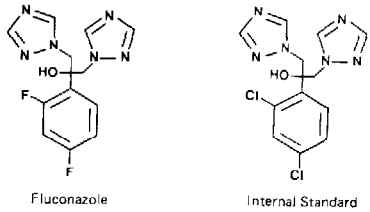

Fig. 1. Structures of fluconazole and internal standard.

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Street et al. [4] reported that the adsorption of polar and nitrogenous compounds on GC columns and packings is considerably reduced following pre-treatment of the columns with benzoyl chloride. Using this technique these authors achieved successful assays for morphine and cyclobarbital without the need for derivatisation procedures. Sensitivity of these assays was increased a 1000-fold over that obtained using untreated columns.

The work reported here shows that the pre-treatment of GC columns and packings with benzoyl chloride can be used to enable the development of sensitive assay methods for fluconazole and a series of triazolyl and imidazolyl antifungal drugs, in plasma and urine of laboratory animals and man.

EXPERIMENTAL

Materials and reagents

SE-52 silicone gum was obtained from Perkin-Elmer (Buckinghamshire, U.K.). Chromosorb G NAW, 80—100 mesh, was obtained from Chromatography Services (Wirral, U.K.). Ethyl acetate (BDH, Poole, U.K.) was redistilled over molecular sieve 4A (Fisons, Loughborough, U.K.). All other chemicals were Analar-grade reagents. Fluconazole (UK-49,858; 2-(2,4-difluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)propan-2-ol; Fig. 1) and internal standard (UK-47,265; 2-(2,4-dichlorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)propan-2-ol; Fig. 1) were obtained from Pfizer Central Research (Sandwich, U.K.).

Apparatus

A gas chromatograph (Hewlett-Packard, Model 5710A) fitted with an electron-capture detector was used. Signals were measured using a reporting integrator (Hewlett-Packard HP 3390A).

Preparation of column packing

A glass column (1 m × 2 mm I.D.) was treated and packed using a modification of the method described by Street et al. [4]. The support (300 g of Chromosorb G NAW, 80—100 mesh) was washed with concentrated hydrochloric acid (six 1-l volumes), boiled for 10 min in the acid, and then washed acid free with demineralised water. After drying, the support was acylated by treatment with a mixture of pyridine (300 g) and benzoyl chloride (200 g). The suspension was stirred thoroughly and then allowed to stand for 48 h. The treated support was washed with acetone until the aroma of pyridine could no longer be detected. The acylated support was then air-dried at 200°C until it was free flowing.

A volume (150 ml) of a solution of SE-52 silicone gum in toluene (10%, v/v) was added to the treated support (100 g), stirred thoroughly and allowed to stand for 48 h. The mixture was then filtered and air-dried at 200°C for 2 h.

Preparation of column

The glass column was cleaned with concentrated hydrochloric acid and washed with demineralised water until acid free. After an acetone rinse the column was dried (100°C) and then filled with a mixture of pyridine and benzoyl chloride (3:2, w/w) ensuring that there were no air bubbles present in

the column. The ends were then capped and the column allowed to stand for 72 h. Following the removal of the acylation mixture, the column was rinsed with toluene and then dried (100°C). A solution of SE-52 silicone gum in methylene chloride (5%, w/v) was drawn into the column by suction for half its length and then slowly moved through to the other end of the column and discarded. The procedure was repeated and, following drainage of excess liquid, the column was dried for 1 h at 100°C followed by overnight drying at 350°C. Carrier gas (argon—methane, 95:5, v/v) was passed through the column continuously during the drying periods.

Conditioning of the column

The column was packed with the treated support and, with carrier gas flowing, was heated at 400°C for 1 h and then overnight at 350°C. After cooling and connection to the detector, the column was heated and maintained at 310°C until a steady baseline was obtained.

For the assay, the column temperature was set at 270°C, the detector temperature at 350°C and the injector temperature at 310°C. Flow-rate of carrier gas was 30 ml/min.

Assay procedure

Standard solutions (0.1 mg/ml) were prepared by dissolving fluconazole and the internal standard (Fig. 1) in ethyl acetate.

Sample volumes of plasma or urine (1 ml) were transferred to screw-capped test tubes (10 ml, Soveril, France), and internal standard solution (5 μl) was added. A 1-ml volume of 1 *M* sodium hydroxide and 3 ml of ethyl acetate were then added and the tubes mixed on a rotary mixer for 5 min (12 rpm). Phases were separated by centrifugation at 1500 *g* for 5 min and the upper organic layer was transferred to clean tubes. The drug was back-extracted into 1 *M* hydrochloric acid (2 ml) from the organic phase by mixing and phase separation as described above. The organic layer was discarded and 5 *M* sodium hydroxide (1 ml) and ethyl acetate (3 ml) were added to the aqueous phase. Mixing and separation was carried out as above. The organic phase was transferred to a tapered screw-capped tube and the contents were evaporated to dryness under a stream of nitrogen at 37°C.

Standard samples were prepared by adding known volumes of fluconazole standard solution to control plasma or urine to give concentrations in the range 0.1—1 μg/ml followed by internal standard solution (5 μl). The standard samples were then extracted according to the procedure described above.

Residues obtained following extraction were re-dissolved in 200 μl of ethyl acetate by mixing on a vortex mixer (5 s). Sample volumes (3 μl) were then injected onto the gas chromatography column.

Calibration curves were derived by plotting the peak-height ratios of drug and internal standard in standard samples against the known concentrations of the drug, using linear regression analysis. Concentrations of fluconazole in unknown samples were then derived by interpolation of the observed peak-height ratio.

RESULTS

Calibration curve

Typical traces of the chromatogram of fluconazole and internal standard are shown in Fig. 2. A typical calibration curve is shown in Fig. 3.

Extraction efficiency

The efficiency of the procedure measured by comparison of peak heights obtained for fluconazole after extraction using the assay procedure with those obtained by direct injection of pure solutions of fluconazole was greater than 90% over the concentration range 0.1—1 μg/ml. Since the pK_a of both fluconazole and internal standard is close to 3, extraction is possible at normal plasma pH without loss of efficiency. However, extraction at basic pH produced cleaner extracts for chromatography.

Accuracy

Prepared samples of plasma and urine containing amounts of drug not known to the analyst were assayed using the procedure. The results (Table I) show that the errors were random over the range of the assay. The recoveries (mean ± S.D.) for plasma (n = 10) and urine (n = 10) were 100 ± 5.3% and 100.6 ± 3.8%, respectively.

Precision

Known volumes of the standard solution of fluconazole were added to samples of control plasma to give six replicates each at concentrations of 0.2,

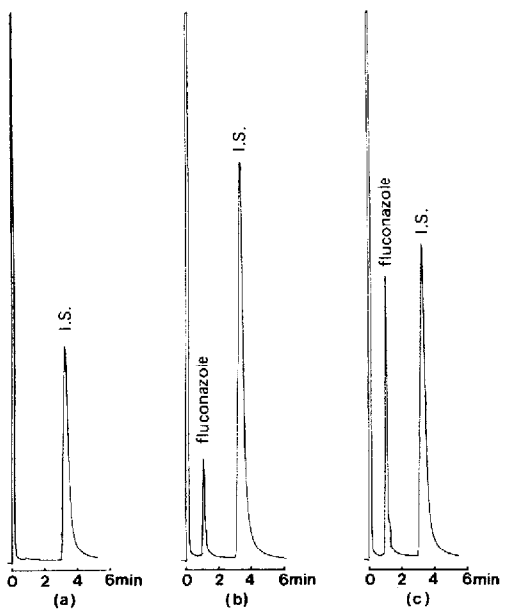

Fig. 2. Gas chromatographic profiles of fluconazole and internal standard (I.S.). (a) Blank plasma extract; (b) 0.1 μg/ml fluconazole; (c) 0.7 μg/ml fluconazole.

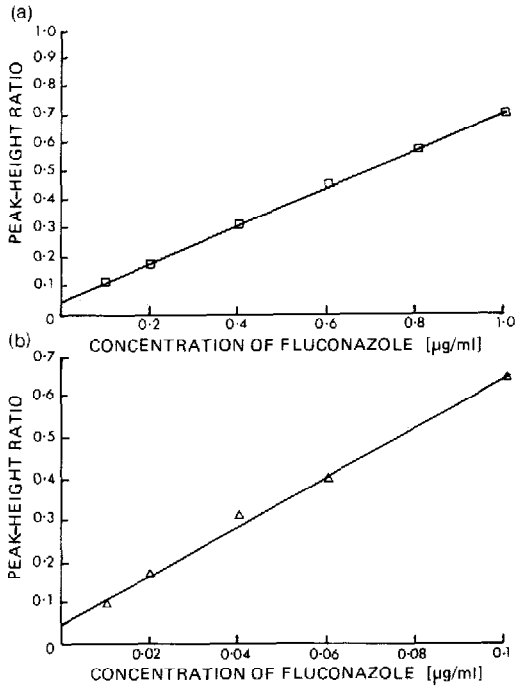

Fig. 3. Calibration curves of fluconazole in human plasma. (a) 0.1—1.0 μg/ml; (b) 0.01—0.1 μg/ml.

TABLE I

RECOVERY OF FLUCONAZOLE FROM PREPARED SAMPLES OF PLASMA AND URINE CONTAINING CONCENTRATIONS OF DRUG NOT KNOWN BY THE ANALYST

Plasma			Urine		
Amount added (μg)	Amount found (μg)	Recovery (%)	Amount added (μg)	Amount found (μg)	Recovery (%)
0.29	0.32	110.3	0.83	0.80	96.4
0.30	0.31	103.3	0.70	0.71	101.4
0.35	0.33	94.3	1.00	1.05	105.0
0.40	0.38	95.0	0.69	0.72	104.3
0.42	0.40	95.2	0.30	0.28	93.3
0.63	0.62	98.4	0.56	0.58	103.6
0.70	0.69	98.6	0.40	0.40	100.0
0.74	0.73	98.6	0.74	0.77	104.1
0.92	0.91	98.9	0.56	0.55	98.2
1.00	1.07	107.0	0.88	0.88	100.0
Mean		100.0			100.6
S.D.		5.31			3.81
95% confidence limit		96.2—103.8			97.9—103.4

0.5 and 0.9 μg/ml. The mean (± R.S.D.) concentrations found were 0.19 (nil), 0.5 (± 2.0%) and 0.9 (± 0.9%) μg/ml, respectively.

Profile in plasma

A typical profile of plasma concentrations in a human volunteer, determined using the method, following a single oral dose of fluconazole in the expected therapeutic range (1.0 mg/kg) is shown in Fig. 4.

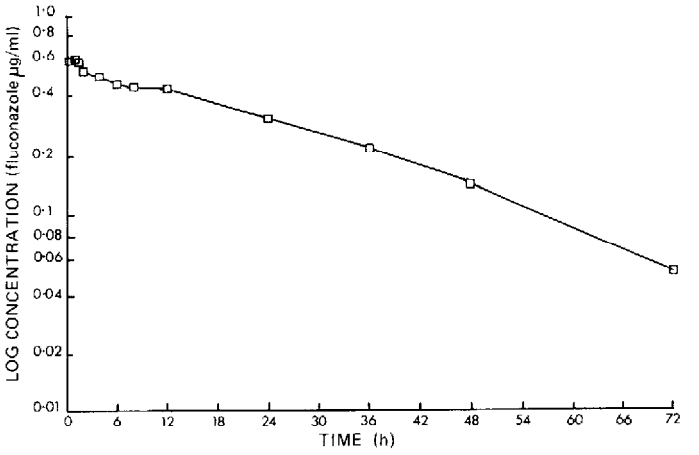

Fig. 4. Concentrations of fluconazole from plasma in a male human volunteer following administration of a single oral dose (0.5 mg/kg) as a solution.

DISCUSSION

Fluconazole, a triazolyl antifungal agent, is representative of an important new class of drugs for clinical use in superficial and systemic fungal infections. These triazole drugs have been shown to exhibit high in vivo potency, allowing the use of dose levels in the range 0.1–1 mg/kg in a number of animal models of fungal infection. [1, 5]. Recent studies with fluconazole in man [6] have shown that effective doses for therapy of vaginal candidosis are equivalent to 0.75 mg/kg per day for three days. At this dose level peak concentrations of fluconazole are less than 2 μg/ml. In common with several imidazoles and other triazole drugs in this class, the in vitro activity (bioassay) of fluconazole is variable and is strongly influenced by medium composition [5, 7]. Thus the sensitivity of any bioassay is dependent upon both the culture conditions and the test organisms used. Consequently bioassay may not be practicable over the range of concentrations likely to be observed in therapeutic use and a sensitive chemical assay will be required to monitor blood levels.

The UV absorption of fluconazole is relatively weak at wavelengths suitable for detection using conventional high-performance liquid chromatography mobile phases; consequently, with currently available equipment, this method could not provide the required sensitivity to conduct pharmacokinetic studies at the effective dose level. Since the compound contained a halogenated phenyl group and two nitrogen heterocycles, GC using electron-capture or nitrogen detection appeared to be the most suitable means of developing a sensitive

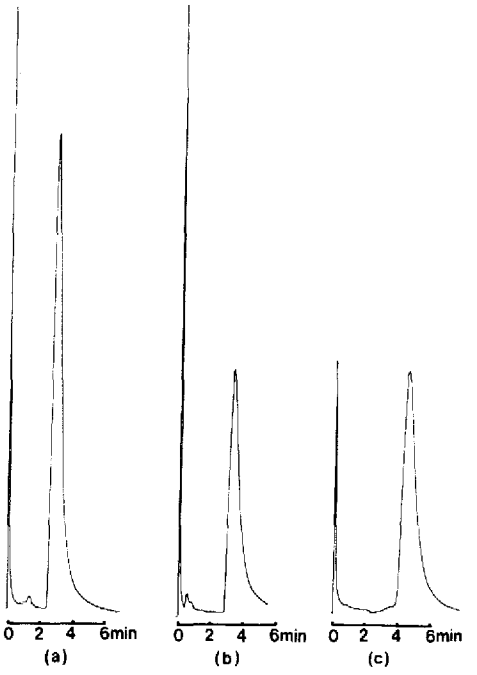

Fig. 5. Gas chromatographic profiles of (a) tioconazole, (b) econazole and (c) miconazole. Injector temperature 320°C, column temperature 280°C and detector temperature 350°C.

assay to analyse a large number of samples. Initial tests indicated that electron-capture detection was more sensitive than nitrogen detection for this compound. Nitrogenous compounds have proved to be difficult to assay at sub-microgram concentrations using conventional GC columns [8], poor peak shape and tailing presumably being due to the adsorption of nitrogen-containing moieties on active sites on the column and packing. In addition the structure of fluconazole does not lend itself to facile derivatisation procedures to overcome adsorption. Derivatisation of the tertiary alcohol group is possible, but only under conditions which are not suitable for a high sample throughput. A modification of the method of deactivating GC columns described by Street et al. [4] was, therefore, used which enabled the development of an assay with a limit of detection of 0.1 μg/ml. Initial pharmacokinetic studies in volunteers (Fig. 4) showed that this was adequate for therapeutic monitoring. This assay method has been shown to be capable of significantly greater sensitivity and has been validated down to concentrations as low as 10 ng/ml in plasma for use in animal experiments in our laboratories. Columns prepared in this way can also be used, with suitable adjustments in column temperature, for the assay of a number of experimental and clinical azole antifungal drugs, which also contain dichlorophenyl or difluorophenyl groups, including tioconazole, econazole and miconazole (see Fig. 5) with similar ranges of sensitivity.

REFERENCES

1 K. Richardson, K.W. Brammer, M.S. Marriott and P.F. Troke, Antimicrob. Agents Chemother., 27 (1985) 832.

2 M.J. Humphrey, S. Jevons and M.H. Tarbit, Antimicrob. Agents Chemother., 28 (1985) 648.
3 F.C. Odds, J. Antimicrob. Chemother., 6 (1980) 749.
4 H.V. Street, W. Vycudilik and G. Machata, J. Chromatogr., 168 (1979) 117.
5 P.F. Troke, R.J. Andrews, K.W. Brammer, M.S. Marriott and K. Richardson, Antimicrob. Agents Chemother., 28 (1985) 851.
6 S. Jevons and M.H. Tarbit, Proceedings of 11th ISHAM Congress, Atlanta, GA, May 19—24, 1985, Abstract R2-3.
7 K. Richardson, R.J. Andrews, M.S. Marriott, M.H. Tarbit and P.F. Troke, in K. Iwata and H. van den Bossche (Editors), In Vitro and in Vivo Evaluation of Antifungal Agents, Elsevier, Amsterdam, 1986, p. 147.
8 C. McMartin and H.V. Street, J. Chromatogr., 22 (1966) 274.