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Gas chromatographic and high-performance liquid chromatographic methods for the determination of genaconazole in biological fluids

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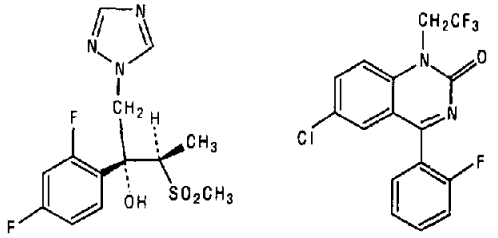
Abstract

Gas chromatographic (GC) and high-performance liquid chromatographic (HPLC) methods were developed for the determination of genaconazole in biological fluids. Both methods involved organic solvent extraction followed by solid-phase extraction on a C_{18} column. GC analysis utilized a megabore column (DB-17) with ^{63}Ni electron-capture detection, whereas HPLC analysis utilized separation on a reversed-phase column with a methanol–phosphate buffer mixture as the mobile phase and quantitation by UV absorbance at 208 nm. Both methods yielded good linearity, accuracy and precision. The limit of quantitation (LOQ) was 0.025 μg per ml of serum for GC and 0.2 μg per ml of serum or 0.5 μg per ml of urine for HPLC analysis. Both GC and HPLC methods were used for the determination of serum concentration–time curves of genaconazole in man following oral administration of a 50-mg dose.

1. Introduction

Genaconazole, (±)-(2R^*,3R^*)-2-(2,4-difluorophenyl)-3-methylsulfonyl-1-(1,2,4-triazole-1-yl)-butan-2-ol (SCH 39304) (Fig. 1), is a novel potent triazole antifungal agent which has shown activity both orally and topically. Genaconazole is active *in vitro* against yeast and dermatophytes. *In vivo* it is superior to ketoconazole and fluconazole in a *Candida* systemic infection model in normal and compromised mice, and in a vaginal *Candida* model in normal hamsters. It is also more active than fluconazole and ketoconazole in a *Trichophyton* dermatophyte model in guinea pigs.

Initially a simple high-performance liquid chromatographic (HPLC) method was developed to facilitate the pharmacokinetic evaluation of genaconazole in animals. This method was used for the determination of genaconazole



GENACONAZOLE SCH 18778

Fig. 1. Chemical structures of genaconazole and SCH 18778 (internal standard)

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in animal plasma and urine after an oral dose of 20 mg/kg. However, the limit of quantitation (LOQ) of 0.2 μg/ml for the HPLC method may not be adequate for the determination of serum concentrations in man following an oral dose of 50 mg or less. Therefore, a more sensitive gas chromatographic–electron capture detection (GC–ECD) method using a megabore column was developed for the determination of genaconazole in human serum to facilitate pharmacokinetic studies in man. This report describes both the HPLC and GC–ECD procedures for the analysis of serum concentrations of genaconazole.

2. Experimental

2.1. Compounds

Glass-distilled ethyl acetate, ether and methanol were obtained from Mallinckrodt (Paris, KY, USA). Authentic standards of genaconazole and 6-chloro-1,2-dihydro-4(O-fluorophenyl)-1-(2,2,2-trifluoroethyl)-quinazoline-2-one, SCH 18778 (internal standard) (Fig. 1) were obtained from Sumitomo (Osaka, Japan) and Schering-Plough (Kenilworth, NJ, USA), respectively.

2.2. Sample preparation

To either 0.5 ml of serum or 0.1 ml of urine, 1.0 ml of water was added. These samples were then extracted with 6 ml of ethylacetate (for GC) or ether (for HPLC) by vortex-mixing for one minute. After centrifugation at 1400 *g* for 10 min, 5 ml of the organic layer were transferred to a clean tube and evaporated to dryness under nitrogen. The residue was then dissolved in 1.5 ml of water and applied to a disposable C_{18} extraction cartridge (J.T. Baker Chemical, Phillisburg, NJ, USA) which had been prewashed successively with 2 ml of methanol and 2 ml of water under 15–20 kPa vacuum (Speed Mate-30, Applied Separations, Allentown, PA, USA). The extraction column was then eluted with 2 ml of water followed by 1 ml of 10% methanol in water and 1.5 ml of methanol. The methanol fraction was collected and evaporated to dryness under nitrogen. For GC analysis, the residue was dissolved in 200 μl of ethyl acetate containing 0.05 μg of the internal standard, and a 1-μl aliquot of the mixture was injected on column. For HPLC analysis, the residue was dissolved in 0.5 ml of the mobile phase (60 parts of 0.03 *M* phosphate buffer pH 5.5, 26 parts of methanol and 8 parts of acetonitrile) and a 50-μl aliquot of this solution was injected.

2.3. GC analysis

The GC chromatographic unit (Varian Model 3700) was equipped with a ^{63}Ni electron-capture detector and a DB-17 fused-silica megabore column (J and W Scientific, Rancho Cordova, CA, USA), 30 cm × 0.53 mm I.D. with a film thickness of 0.1 μm. The carrier and make-up gases were nitrogen with flow-rates of 5 and 25 ml/min, respectively. The temperatures of the injector, column and detector were 210°C, 245°C and 290°C, respectively. The electrometer attenuation was set at 16–64 and range at 12.

2.4. HPLC analysis

The HPLC system consisted of a Waters Model 6000A pump and a Model 480 absorbance detector set at a wavelength of 208 nm (Waters Associates, Millford, MA, USA). Separation was accomplished on a Waters μBondapak C_{18} column (10 μm, 30 × 0.46 cm I.D.). The absorbance detector output was monitored with a 10-mV recorder and the detector sensitivity was set at 0.01 AUFS. The mobile phase [60 parts of 0.03 *M* phosphate buffer (pH 5.5), 26 parts of methanol and 8 parts of acetonitrile) was delivered at 1.0 ml/min. All separations were carried out at ambient temperature.

2.5. Calculations

Peak-height ratio (for GC) or peak height (for HPLC), retention times and concentration were calculated by an HP-3357 Laboratory Automation System (Hewlett-Packard, Palo Alto, CA, USA). Calculation of the concentrations of

genaconazole was based on either the peak-height ratio of genaconazole to that of the internal standard or the peak height of genaconazole. The slopes, intercepts and correlation coefficients were determined by least square linear regression analysis using a weighing factor of $1/y$.

3. Results

3.1. Serum

Typical GC and HPLC chromatograms of serum samples from volunteers who received an oral dose of 50 mg are shown in Figs. 2 and 3, respectively. The retention times for genaconazole were 9.1 min for GC and 11.5 min for HPLC analysis, respectively. These were clearly different from the retention times of several analogs and other common drugs such as fluconazole, econazole, ketoconazole, miconazole, terconazole, sulconazole, caffeine, chlorpheniramine, pseudoephedrine and acetaminophen. Therefore, the method is found to be specific for genaconazole by both the GC and the HPLC procedures.

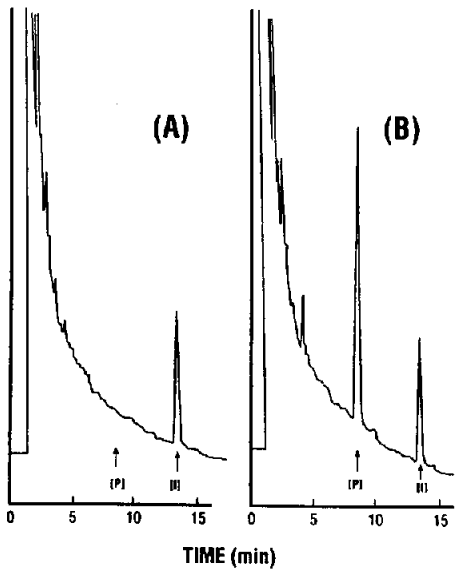

Fig. 2. Typical GC–ECD chromatograms of genaconazole and the internal standard (I) from (A) pre-dose (0 hr) human serum and (B) human serum at 4 hr after oral administration (50 mg) of genaconazole. The y-axis represents the detector response and x-axis represents the retention time.

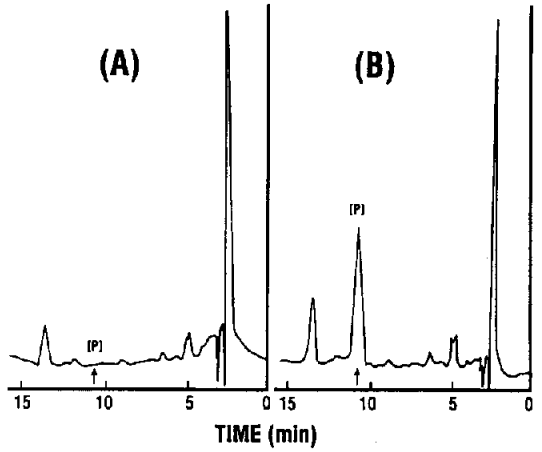

Fig. 3. Typical HPLC chromatograms of genaconazole (P) from (A) pre-dose (0 hr) human serum and (B) human serum at 4 hr after oral administration (50 mg) of genaconazole. The y-axis represents the detector response and x-axis represents the retention time.

The standard curve for genaconazole in the GC procedure was obtained by plotting the ratio of the peak height of genaconazole to the peak height of the internal standard against the serum concentrations (0.025–5 μg/ml) of genaconazole. There was a good linear relationship between the peak-height ratio and serum concentration, as determined by regression analysis of the data using the equation $y = mx + b$, where $m = 0.4764$, $b = 0.0007$ and $r = 0.999$.

The linearity of the HPLC assay was determined by the analysis of serum samples containing 0.2 to 40 μg/ml of genaconazole. Linear regression analysis of the peak heights (y) *vs.* theoretical concentrations (x) gave the following equation: $y = 629x + 83.5$; ($r = 0.998$). The correlation coefficient ($r = 0.998$) demonstrated the excellent linearity of the method over the concentration range studied.

The intra-assay precision of the two methods was determined using three concentrations of genaconazole in serum for GC (0.025, 0.2 and 1.0 μg/ml) and HPLC (0.2, 2 and 10 μg/ml) analysis, respectively. There were six replicates at each concentration in a single run. The co-

Table 1
Intra-assay precision of the GC and HPLC methods in human serum

Method	Concentration added (μg/ml)	Mean concentration found ($n = 6$) (μg/ml)	(%)
GC	0.025	0.0258	8.9
	0.2	0.205	4.1
	1.0	0.993	3.2
HPLC	0.2	0.211	4.5
	2.0	2.2	4.9
	10.0	9.4	3.7

efficients of variation ranged from 3.2 to 8.9% for both procedures indicating that the two methods were accurate and reproducible (Table 1). The inter-assay precision of the two methods was established by repeated determinations ($n = 5$) on five consecutive days (Table 2). The coefficient of variation for inter-assay precision was 2.1% at 0.5 μg/ml for GC and 1.8% at 10 μg/ml for HPLC analysis, respectively.

The limit of quantitation (LOQ) of the method was 0.025 μg/ml (C.V. = 8.9%) for GC and 0.2 μg/ml (C.V. = 4.5%) for HPLC analysis, respectively.

3.2. Urine

The HPLC analysis was the only method validated for the determination of genaconazole in urine. Chromatograms of human urine extracts following oral administration of 50 mg dose of genaconazole are shown in Fig. 4.

The calibration curve for concentrations of genaconazole in urine ranged from 0.5 to 40 μg/ml. The linearity was determined by plotting the peak height (y) *vs.* theoretical concentration (x). Linear regression analysis gave an equation of $y = 499.3x + 13.9$ ($r = 0.989$).

The intra-assay precision ($n = 6$) of the HPLC method was demonstrated by the low coefficient of variation (3.2% for 2 μg/ml and 1.9% for 10 μg/ml) obtained. Similar results were also obtained for the inter-assay precision ($n = 5$) at 10 μg/ml with a coefficient of variation of 4.4% for analysis on five consecutive days. The limit of quantitation (LOQ) of genaconazole in urine was 0.5 μg/ml, with a coefficient of variation of 2.9% ($n = 6$).

4. Discussion

Although microbiological assays have been used to screen the *in vitro* activity of several

Table 2
Inter-assay precision of the methods in human serum

Method	Day of assay	Intra-assay variation		Between-day variation	
		Mean ($n = 5$)	C.V.(%)	Mean ($n = 5$)	C.V.(%)
GC	1	0.49	3.6	0.486[a]	2.1
	2	0.49	5.2		
	3	0.47	2.7		
	4	0.48	5.0		
	5	0.49	1.8		
HPLC	1	10.1	2.8	9.98[a]	1.8
	2	9.8	2.1		
	3	9.8	1.5		
	4	10.0	2.5		
	5	10.2	1.6		

[a]Theoretical values: GC = 0.50 μg/ml, HPLC = 10.0 μg/ml.

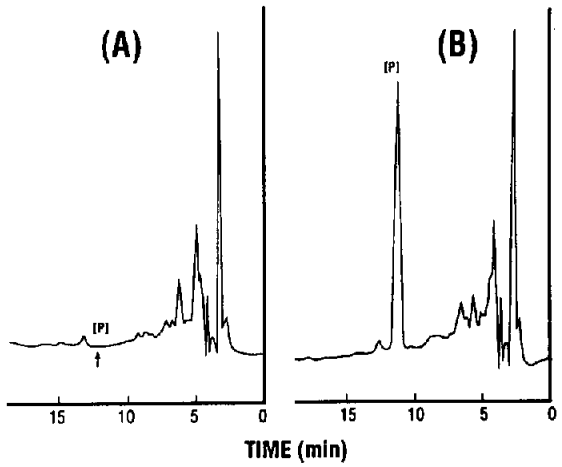

Fig. 4. Typical HPLC chromatogram of genaconazole (P) from (A) pre-dose (0 hr) human urine and (B) human urine (0–6 hr) after oral administration (50 mg) of genaconazole. The y-axis represents the detector response and x-axis represents the retention time.

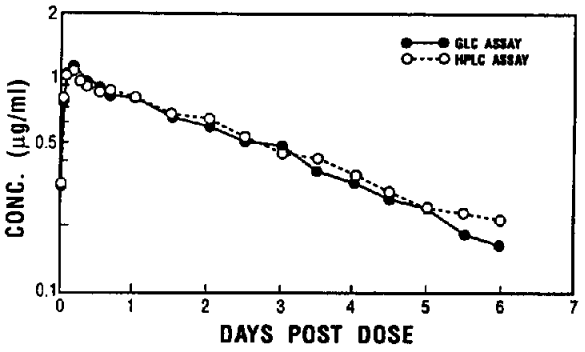

Fig. 5. Mean serum concentrations of genaconazole in man after oral administration of a 50 mg dose determined by the HPLC and GC–ECD methods.

imidazole drugs, these assays were too variable and also strongly influenced by media composition and the test organisms used to be suitable for pharmacokinetic evaluation of these drugs [1,4]. Alternatively, more specific assays, such as HPLC and GC, do not measure activity, such that nonquantifiable amounts of parent drug, or biologically-active metabolites, may be inadvertently omitted in analysis and interpretation of therapeutic data.

Serum concentrations of genaconazole were determined in animal studies at high doses by reversed-phase HPLC analysis with detection at 208 nm and an LOQ of 0.2 μg/ml. This method could not provide the required sensitivity to conduct clinical pharmacokinetic studies at the low therapeutic doses anticipated. GC assays for polar nitrogen compounds [2,3] and several triazole antifungal drugs [5] with pretreatment of the column packing with benzoyl chloride were developed. However, this acylation procedure is very tedious and time-consuming. Since the analyte compound contains two halogens in its molecular structure (Fig. 1), GC using electron-capture detection appeared to be the most suitable means of developing a very sensitive assay.

In the present study, we developed an GC–ECD method with a megabore column. The method had an LOQ of 0.025 μg/ml of serum. The utility of the HPLC and GC–ECD methods for the determination of genaconazole in clinical pharmacokinetic studies was established by the analysis of serum and urine samples from two volunteers who each received a single oral dose of 50 mg. Urine was determined to be the main route of excretion, and the cumulative amount of unchanged genaconazole excreted in urine accounted for 65% of the dose. Comparable serum concentration–time curves were obtained using both the HPLC and the GC methods (Fig. 5). Regression analysis of the data from the GC–ECD method (y) *vs.* data from HPLC method (x) yielded equation $y = 0.9254x + 0.04819$ ($r = 0.998$). Thus, the data obtained from the two methods were well correlated at serum concentrations greater than 0.2 μg/ml. However, when serum concentrations were less than 0.2 μg/ml, the correlation between the two methods, as expected, was poor. This was probably due to the low intrinsic sensitivity (LOQ = 0.2 μg/ml) of the HPLC method, which is approximately eight times less sensitive than the GC–ECD method (LOQ = 0.025 μg/ml).

5. Conclusion

The GC–ECD and HPLC methods for the determination of genaconazole in human serum

are specific, sensitive, accurate and reproducible. The HPLC method was shown to be useful for determining serum concentrations following oral administration of 100 mg or higher, while the GC–ECD method was suitable for oral administration of 100 mg or lower. Therefore, both methods can be utilized for clinical pharmacokinetic evaluation of the drug. In addition, the HPLC method was suitable for urine analysis.

6. References

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