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SIMULTANEOUS DETERMINATION OF GRISEOFULVIN AND 6-DESMETHYLGRISEOFULVIN IN PLASMA BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

Two methods have been developed for the simultaneous determination of griseofulvin and its major metabolite 6-desmethylgriseofulvin in plasma using electron-capture gas chromatography. The first method was based on the quantitative reversion of the 6-desmethyl metabolite to griseofulvin by diazomethane. Plasma extract was chromatographed both before and after treatment with diazomethane, the former being the measure of griseofulvin and the latter representing the sum of the two compounds. In the second method, plasma extract was treated with diazobutane and griseofulvin and the butylated metabolite were separated by gas chromatography. The sensitivity for griseofulvin was 20 ng/ml by both methods and that for the metabolite was 20 ng/ml and 40 ng/ml by the first and the second method, respectively. The concentrations of the metabolite as well as griseofulvin were determined in dog and human plasma after oral administration of griseofulvin.

INTRODUCTION

Griseofulvin is an orally active antifungal agent used widely in clinical practice. The concentration of this drug in blood has been measured by spectrofluorometry [1, 2], electron-capture gas chromatography [3] and, more recently, high-performance liquid chromatography [4, 5]. In all of these methods, the concentration of unmetabolized griseofulvin has been the object of determination. Since griseofulvin is metabolized extensively to 6-desmethylgriseofulvin in rabbits [6], dogs [7] and man [8, 9] and excreted in urine almost exclusively as the 6-desmethyl metabolite and its conjugate, the de-

termination of 6-desmethylgriseofulvin in blood in addition to the unchanged drug should be useful in biopharmaceutical studies. This report describes gas chromatographic (GC) methods of determining griseofulvin and 6-desmethyl-griseofulvin simultaneously in blood plasma.

EXPERIMENTAL

Chemicals

Griseofulvin was purchased from Nihon Kayaku (Tokyo, Japan). Tablets each containing 125 mg of griseofulvin, were a commercial preparation (Yamanouchi Pharmaceutical, Tokyo, Japan). 6-Desmethylgriseofulvin was isolated from urine of a male beagle dog, which received orally a total of 12 g of griseofulvin, according to the method of Harris and Riegelman [7]. The compound was recrystallized from ethanol and had a melting point of 276—278° (decomp., lit. 279—281° [7]). The IR spectrum agreed with that reported in the literature [7]. The elemental analysis and NMR spectrum also supported the structure.

Indomethacin methyl ester, used as the internal standard, was prepared from indomethacin (Sumitomo Chemical, Osaka, Japan). A 0.5—1-ml aliquot of dichloromethane containing 1 mg of indomethacin was mixed with 3 ml of ethereal diazomethane and left at room temperature for 5 min. After evaporation of the reagent with slight warming, the residue was dissolved in benzene to a concentration of 100 μg/ml. This solution was stable for at least one month at room temperature. At the time of assay, an aliquot was diluted with methanol to give a concentration of 1.25 μg/ml. Ethereal solution of diazomethane was prepared from *p*-toluenesulfonyl-N-methyl-N-nitrosamide (Tokyo Kasei, Tokyo, Japan) using a specialized equipment Diazald® Kit (Aldrich, Milwaukee, Wisc., U.S.A.). Ethereal solution of diazobutane was prepared simply as follows. To 0.8 g of N-butyl-N-nitrosourea (Nakarai, Kyoto, Japan), dissolved in 8 ml of diethyl ether and cooled to 0°, was added 3 g of KOH pellets. After 10 min of occasional shaking, the diethyl ether layer was transferred to another glass tube containing 2 g of KOH pellets and was used for derivatization. All other reagents used were commercial preparations and of analytical grade.

Instrumentation

A Hewlett-Packard Model 5710A gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The column was a glass tube (110 cm × 1.8 mm I.D.) packed with 1.5% OV-225 on 100—200 mesh Gas-Chrom Q (Nihon Chromato Works, Tokyo, Japan). The temperatures were 300° for the injector, 275° for the column, and 300° for the detector. The carrier gas, argon—methane (95:5), was dried over molecular sieve and passed at a flow-rate of 30 ml/min. Mass spectra were obtained with a Hitachi RMU-6MG combined gas chromatograph—mass spectrometer under the following conditions: electron energy 20 eV, emission current 80 μA, ion source temperature 160°, accelerating voltage 1.4 kV.

Simultaneous determination of griseofulvin and 6-desmethylgriseofulvin

Method 1. 6-Desmethylgriseofulvin in the plasma extract was converted to and assayed as griseofulvin after griseofulvin itself had been assayed with the same extract. To 0.5 ml of plasma were added 1.5 ml of 0.1 *N* hydrochloric acid and 0.2 ml of methanol containing 250 ng of indomethacin methyl ester. The mixture was extracted with 5 ml of diethyl ether and, after centrifugation, the diethyl ether layer was taken to dryness at 40—50°. The residue was dissolved in 0.1 ml of benzene and 1—2 μl was injected into the gas chromatograph column for the determination of the unchanged drug. The remaining benzene solution was then treated with 0.2 ml of ethereal diazomethane and, after leaving for 5 min at room temperature, evaporated. This treatment converted the 6-desmethyl metabolite quantitatively to griseofulvin [8]. After reconstituting the residue in 0.1 ml of benzene, 1—2 μl was chromatographed for the determination of the unchanged drug plus its metabolite. At a column temperature of 275°, the retention times of the internal standard and griseofulvin were 3 min and 3.8 min, respectively.

Alternatively, the diethyl ether layer was divided into two parts and each was evaporated to dryness. The residue from one part was taken up in 50 μl of benzene and that from the other half was treated with 0.1 ml of ethereal diazomethane, followed by evaporation after 5 min.

The amount of griseofulvin as well as griseofulvin plus its metabolite was calculated by measuring the peak height ratio of griseofulvin and the internal standard and referring to the standard curve. The standard curve was prepared by subjecting to the above procedure 0.5 ml of the drug-free control plasma, to which 10—250 ng of griseofulvin dissolved in 0.2 ml of 20% methanol had been added. The peak height ratio of griseofulvin and the internal standard was plotted against the concentration of griseofulvin. When the concentrations of the two compounds were high, the plasma samples were appropriately diluted with distilled water.

Method 2. Extraction of the two compounds from plasma was performed as in Method 1. After evaporation of the diethyl ether layer, 0.2 ml of etheral diazobutane was added to the residue. The mixture was left at room temperature for 5 min, the reagent evaporated and the residue dissolved in 0.1 ml of benzene. A 1—2-μl volume of the solution was injected into the column. At a column temperature of 275°, the retention times of the internal standard, griseofulvin and butylated 6-desmethylgriseofulvin were 3.0 min, 3.8 min and 5.2 min, respectively. Ratios of the peak height of griseofulvin as well as the butylated metabolite to that of the internal standard were measured. Standard curves were prepared by analyzing drug-free control plasma spiked with 10—250 ng each of the two compounds per 0.5 ml according to the above procedure.

Extraction recoveries

To 0.5 ml of control dog plasma were added 200 ng of griseofulvin or the metabolite dissolved in 0.2 ml of 20% methanol. After adjusting the pH between 0.5 and 10 by the addition of 0.1—1.0 *N* HCl or 0.1—1.0 *N* NaOH and bringing the volume to 2 ml, the mixture was extracted with 5 ml of diethyl ether, followed by centrifugation. A 2.5-ml aliquot of the diethyl ether layer

was mixed with 200 ng of the internal standard dissolved in 0.5 ml of diethyl ether and analyzed for each compound by Method 1. The extraction recoveries were calculated by comparing the peak height ratios with those obtained when 100 ng of each compound, dissolved in 2.5 ml of diethyl ether, was mixed with 200 ng of the internal standard and processed without the extraction procedure.

Animal and human studies

Three male beagle dogs, weighing 8—12 kg, were fasted for 21 h and given 125 mg of griseofulvin orally as a tablet. Similarly, three male volunteers, aged 38—44 years, received 250 mg of the drug after overnight fasting. Blood samples were obtained by venipuncture with heparinized syringes and immediately centrifuged. Plasma samples were stored frozen until analyzed.

RESULTS AND DISCUSSION

Extraction of griseofulvin and the metabolite from plasma

6-Desmethylgriseofulvin differed from griseofulvin in its extractability from plasma. While the metabolite was extracted quantitatively with diethyl ether below pH 4, it was extracted less efficiently at higher pH values and remained totally unextracted above pH 8. Similar results have previously been reported for the metabolite in urine by Rowland and Riegelman [10]. Extraction of griseofulvin, on the other hand, was quantitative at any pH value (Fig. 1).
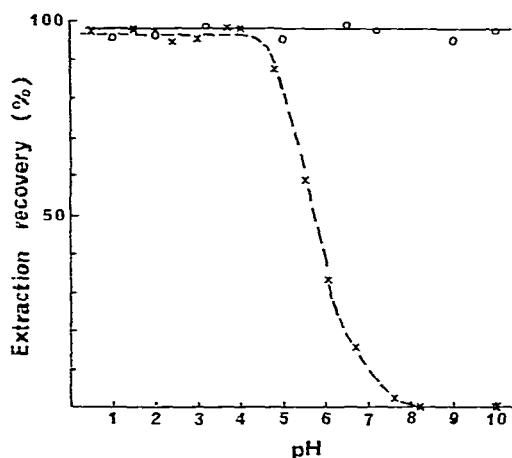

Fig. 1. Effect of pH on the extraction of griseofulvin (O——O) and 6-desmethylgriseofulvin (×- — -×) from plasma.

Evaluation of the methods

In the preliminary study in which GC properties of 6-desmethylgriseofulvin were examined using various columns and operating conditions, it was found that this metabolite does not produce a well-defined chromatographic peak when analyzed without derivatization, as reported by Schwarz et al.

[3]. The phenol function of the metabolite had to be derivatized for successful chromatography. Trimethylsilylation, which was used by Kabasakalian et al. [11] to measure the metabolite in human urine, was not quite satisfactory since the chromatographic peak tended to be accompanied by tailing and separation from endogenous material in plasma was sometimes difficult. Acylation of the phenol group, particularly propionylation, produced a derivative with a fine chromatographic property and which was separated from griseofulvin and endogenous material using a column of 3% OV-105. The drawback of the propionylated metabolite was its much lower sensitivity towards electron-capture detection compared with the parent drug and its comparative instability which necessitated careful handling during analysis.

The most satisfactory derivatives in terms of stability, chromatographic properties and simplicity in preparation were obtained by alkylation. 6-Desmethylgriseofulvin was alkylated quantitatively to its methyl and butyl derivatives by the methods described in Experimental. This was confirmed as follows: (1) When the alkylation reaction mixture containing the metabolite (20 μg) and diazomethane or diazobutane was examined by thin-layer chromatography using a silica gel plate (Merck, Darmstadt, G.F.R., silica gel 60 F_{254}) and a solvent system chloroform—diethyl ether—acetone—acetic acid (65:20:15:0.5), the metabolite, detectable by short-wave UV light at R_F 0.37, disappeared within 1 min and was completely replaced by new compounds which appeared at R_F values of 0.59 and 0.71, respectively. The former agreed with griseofulvin in its R_F value. The latter was supposedly a butylated analogue of the drug. (2) GC examination of the reaction mixture revealed that, after treatment with diazomethane, the metabolite gives a well-shaped peak at the same retention time as griseofulvin, while the metabolite itself does not give rise to any discernible peak. An equimolar quantity of the drug and the metabolite (10—250 ng), added either to the alkylation reaction mixture or to control plasma and processed as in Method 1, produced exactly the same peak height demonstrating the quantitative methylation of the latter. After treatment with diazobutane, the metabolite gave a sharp peak at a retention time different from that of the parent drug. The mass spectrum of this peak, obtained by GC—mass spectrometry, showed a molecular ion at m/e 394 in agreement with the supposed structure, while griseofulvin itself showed a molecular ion at m/e 352. The peak height of the butylated metabolite was about 40% less than that from equimolar griseofulvin under the conditions described in Method 2.

The chromatograms obtained from a control dog plasma to which both compounds had been added at a concentration of 200 ng/ml are shown in Fig. 2. As is evident, the peak height of griseofulvin relative to that of the internal standard was doubled when the plasma extract was treated with diazomethane in Method 1. Treatment with diazobutane resulted in appearance of a new peak due to the butylated metabolite. Indomethacin methyl ester as an internal standard, in combination with an OV-225 column, gave excellent separation of the peaks of interest with minimum tailing. Drug-free control plasma gave no interfering peaks either in Method 1 or in Method 2.

The standard curves are shown in Fig. 3. Linear response was obtained for griseofulvin over the concentration range of 20—500 ng/ml plasma in
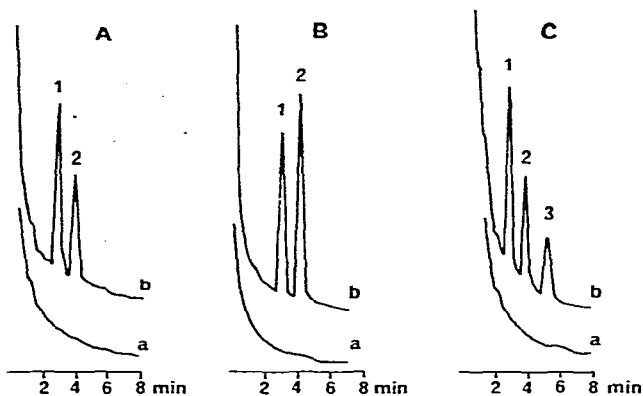
Fig. 2. Chromatograms of control dog plasma (a) and dog plasma spiked with 200 ng/ml each of griseofulvin and 6-desmethylgriseofulvin (b). A, Without derivatization (Method 1); B, after treatment with diazomethane (Method 1); C, after treatment with diazobutane (Method 2). Peaks: 1, internal standard; 2, griseofulvin; 3, butylated 6-desmethylgriseofulvin.
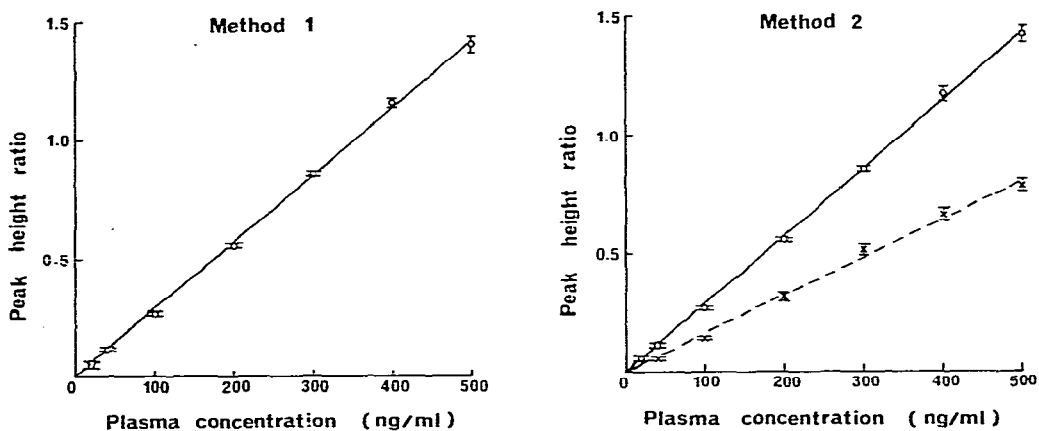

Fig. 3. Standard curve for the determination of griseofulvin (O——O) and 6-desmethylgriseofulvin (X- - -X) in plasma. Each point represents the mean ± S.E.M. from four experiments.

both methods using a 0.5-ml sample. The sensitivity for the metabolite in the second method was slightly lower than that for the parent drug and linear response was obtained over the range of 40—500 ng/ml. The standard curve prepared from 6-desmethylgriseofulvin in the first method was exactly the same as that prepared from griseofulvin. Moreover, by analyzing plasma samples which contained graded amounts of the drug (20—400 ng/ml) in the presence of a fixed amount of the metabolite (200 ng/ml) or plasma samples which contained graded amounts of the metabolite (20—400 ng/ml) in the presence of a fixed amount of the drug (200 ng/ml), it was confirmed that the amount of the drug detected is not affected by the metabolite present when treatment with diazomethane is omitted and the amount detected after diazomethane treatment is exactly equal to that predicted from quantitative conversion of the metabolite.

The advantage of the first method is that it is applicable even when the standard sample of the 6-desmethyl metabolite is not available, while the second method, being a simpler one-step procedure, is preferred when the metabolite is available.

Application of the methods

The two compounds in plasma were determined after oral administration of griseofulvin to dogs and man. All the plasma samples were assayed by both Method 1 and Method 2. The results, obtained by Method 2, are shown in Figs. 4 and 5. An example of a chromatogram is shown in Fig. 6. The plasma concentration of griseofulvin in man was comparable to that of the metabolite throughout the sampling period, which agreed with the result reported by Lin et al. [9] using the ^{14}C-labelled drug. In contrast, the concentration of the metabolite was more than 6 times higher than that of the parent drug

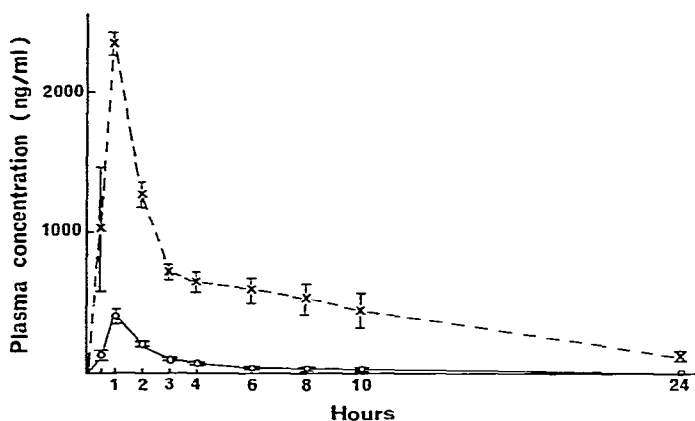Fig. 4. Plasma concentration of griseofulvin (O——O) and 6-desmethylgriseofulvin (×– – –×) in dogs after oral administration of 125 mg of griseofulvin. Each point represents the mean ± S.E.M. from three experiments.
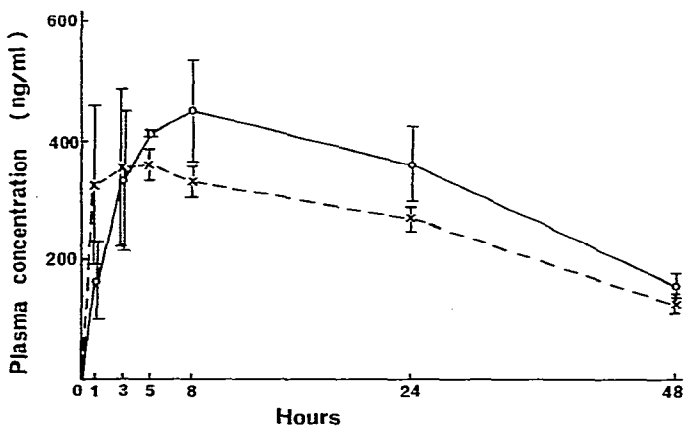

Fig. 5. Plasma concentration of griseofulvin (O——O) and 6-desmethylgriseofulvin (×– – –×) in man after oral administration of 250 mg of griseofulvin. Each point represents the mean ± S.E.M. from three experiments.
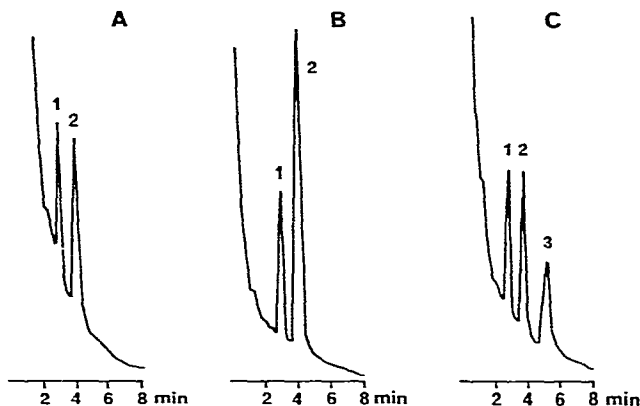
Fig. 6. Chromatograms of a plasma sample obtained from a volunteer 5 h after oral administration of 250 mg of griseofulvin. A, Without derivatization (Method 1); B, after treatment with diazomethane (Method 1); C, after treatment with diazobutane (Method 2). Peaks: 1, internal standard; 2, griseofulvin; 3, butylated 6-desmethylgriseofulvin.
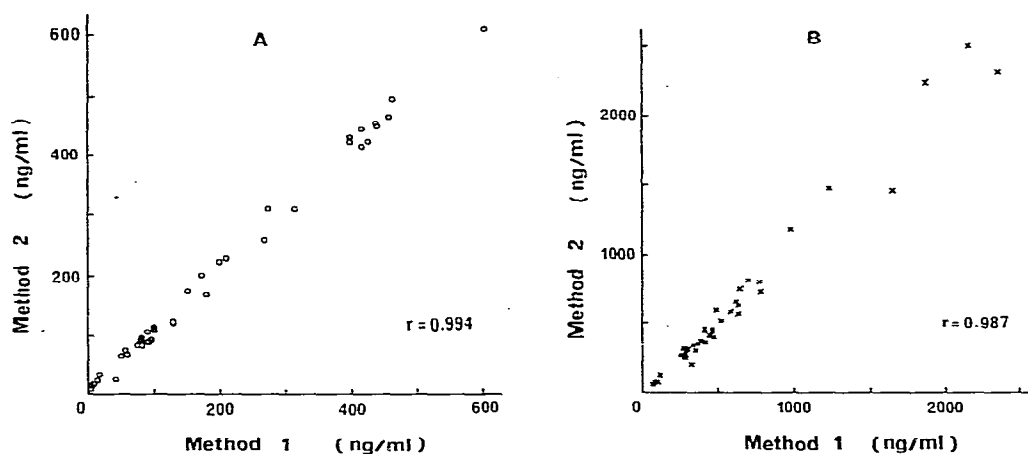

Fig. 7. Correlation between values obtained by Method 1 and Method 2. A, Griseofulvin; B, 6-desmethylgriseofulvin.

in dogs. This observation supports the conclusion reached by Harris and Riegelman [7] on the basis of pharmacokinetic studies that the metabolism of the drug is much faster in dogs than in man. The values obtained for the same samples by the two methods agreed quite well. This is shown in Fig. 7, in which all the data obtained in dogs and man by Method 2 are plotted against the corresponding data obtained by Method 1. The correlation coefficients for the parent drug and the metabolite were 0.994 and 0.987, respectively.

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