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Note

Determination of griseofulvin in rat plasma by high-performance liquid chromatography and high-performance thin-layer chromatography

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For the study of the resorption of different administration forms of griseofulvin (7-chloro-2′,4,6-trimethoxy-6′-β-methylspiro-(benzofuran-2-(3H)-1′-(2)-cyclohexene)-3,4′-dione) a specific and sensitive method of determination for this antifungal antibiotic is required. Spectrofluorimetric methods are sensitive [1—4], but they also detect other metabolites, and they have been substituted by gas-chromatographic methods [5, 6] and a thin-layer chromatographic (TLC) process [7] (self-streaked Al_2O_3/SiO_2 plates with fluorescence detection). Bailey [8] has published a high-performance liquid chromatographic (HPLC) method with ultraviolet detection, but this is not sensitive enough. All these methods require the extraction of griseofulvin from plasma.

This paper describes a sensitive HPLC method using fluorescence detection, with a simplified extraction process, and a simple and effective high-performance TLC (HPTLC) method, also with fluorescence detection.

METHODS

HPLC method

Extraction of griseofulvin from plasma. One millilitre of plasma, diluted with 17 ml of distilled water, is extracted with 60 ml of peroxide-free diethyl ether (technical grade) through an Extrelut^{R}-Fertigsäule (Merck, No. 11737, Nachfüllpackung No. 11738). The eluate is evaporated to dryness at 40° and the residue dissolved in 1—5 ml methanol (p.a. grade) in water (50:50), according to the expected griseofulvin content. The performance of this extraction was tested with rat plasma spiked with different quantities of griseofulvin (0.5, 1.0, 5.0, 10.0 and 100.0 μg/ml).

Deproteinisation of plasma. One millilitre of plasma and 2 ml of ethanol (p.a. grade) are mixed, centrifuged and the supernatant is used for analysis.

Analytical procedure. The reagents used were p.a. grade from Merck (Darmstadt, G.F.R.). The column (10 cm × 3 mm I.D.) was filled with LiChrosorb RP-8 (Merck) or Nucleosil C_8 (Macherey, Nagel & Co., Düren, G.F.R.), particle size 5 μm, column temperature 20°. The fluorescence detectors were a Perkin-Elmer LC 1000 (λ_{exc} = 297 nm (filter), λ_{em} = 428 nm, scale expansion ×20); and a Perkin-Elmer 204, with an adjustable flow-through cell (Hellma, No. 176.70 QS), λ_{exc} = 295 nm, λ_{em} = 428 nm, cut-off filter FL 39 (Zeiss), respectively. Sensitivity control 12, selector ×10. The eluent was acetonitrile—water (40:60); the pump an Orlita DMPAE 10.4, providing a flow of 1.2 ml/min, at a pressure of 180 bar. The injector was a modified 7671 A-Automatic Sampler [9] (Hewlett-Packard) with a pneumatic sample injection valve 900048 L (Latek, Heidelberg, G.F.R.), and a 25-μl loop, filling with a peristaltic pump Mini S 820 (Ismatec).

The standard solution, according to the concentration expected, was 1.0 or 10.0 μg griseofulvin per ml methanol (50%). The standard was pure griseofulvin (Biochemie Ges.m.b.H., Kundl, Austria). Injection mode: after four samples one standard is injected. Recorder: Servogor S (Goerz), paper advance 0.5 cm/min. Calculations were made using the Laboratory Data System 3352 C from Hewlett-Packard.

Under these conditions the retention time of griseofulvin was 5 min.

HPTLC method

The reagents were p.a. grade from Merck. The plates were HPTLC-Fertigplatten Kieselgel 60 für die Nano-DC, 10 × 20 cm (Merck, No. 5641); the developing chamber was also from Merck (No. 11622). The solvent was butylacetate—acetone (4:1). Application: 500 nl microcaps (Drummond).

The standard solutions were: (1) 1 μg griseofulvin per ml methanol (50%); (2) 5 μg griseofulvin per ml methanol (50%); (3) 9 μg griseofulvin per ml methanol (50%). Samples A—J were different samples of rat plasma, diluted 1:1 or more with a 1% solution of 2,5-dimethylbenzosulphonic acid (ammoniumsalt, Merck, No. 3469). The solution to be applied should contain 1—9 μg griseofulvin per ml. The application scheme for 500 nl of each standard solution 1—3 and samples A—J was as follows: AB1CD2EFG3HIJAB1CD2EFG3HIJ. The migration distance was 3 cm (= 5 min), giving an R_F of 0.52 for griseofulvin. Drying time was 30 min at 120°.

Scanning conditions were: Zeiss-Chromatogrammspektralphotometer, excitation by Hg lamp St 41 at 295 nm, measuring through cut-off filter FL 39, F/II/10, measuring slit 3.5 mm, slit of monochromator, 1.3 mm, table advance 50 mm/min, paper advance on Servogor S 60 mm/min, scanning at right-angles to the direction of chromatography, to and fro. Fully automated computation [10] (calibration line: peak area against concentration).

RESULTS

The recoveries of griseofulvin added to plasma ranged between 94.0 and 100.0%. Results obtained from 40 samples, containing 6—100 μg of griseofulvin per ml and analysed by HPLC and HPTLC, showed the same results and a coefficient of correlation of 0.99868. For HPLC there was a close linearity

between peak area and concentration between 0.1 and 100 μg/ml, whilst for HPTLC this linearity was valid between 0.5 and 15 μg/ml (see calibration lines in Fig. 1). HPTLC is faster than HPLC, as can be seen in Table I; the peaks of samples, deproteinised by means of ethanol, were unsuitable for evaluation (Fig. 2). Both methods are of identical accuracy (see Table I); of course, when a sample is analysed once on ten different plates instead of ten times on one plate, the coefficient of variation increases to 2.0%.

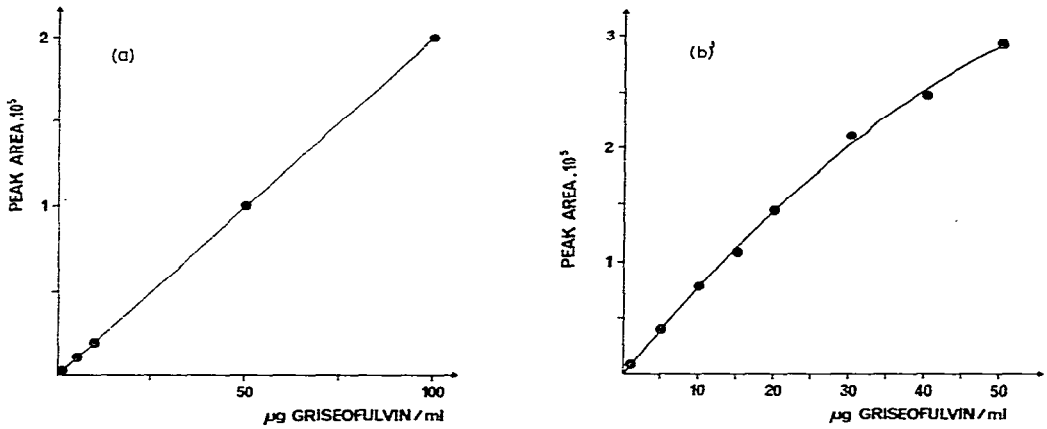

Fig. 1. Calibration curves for HPLC (a) and HPTLC (b).

TABLE I

COMPARISON OF HPLC WITH HPTLC

	HPLC		HPTLC	
Time (min) required for 20 samples	Extraction	270	Dilution	10
	Preparation*	115	Application	20
	Programming	5	Separation	5
	Separation, evaluation	220	Drying	30
			Programming	1
			Measurement adjusting	5
			Scanning, evaluation	25
	Total:	610		96
	Ratio of manipulation	68%		64%
Detection limits (μg/ml, signal-to-noise ratio = 3:1)	LC 1000, λ_{em} = 428 nm	0.1	Zeiss, FL 39	0.15
	204, λ_{em} = 428 nm	0.025		
	204, FL 39	0.06		
Accuracy (C.V. (%), n = 10)		0.8	Analysed on one plate	0.8
			Analysed on ten different plates	2.0

*The preparation time includes in proportion column filling, column testing, services to the apparatus and functional control of the analysis equipment.

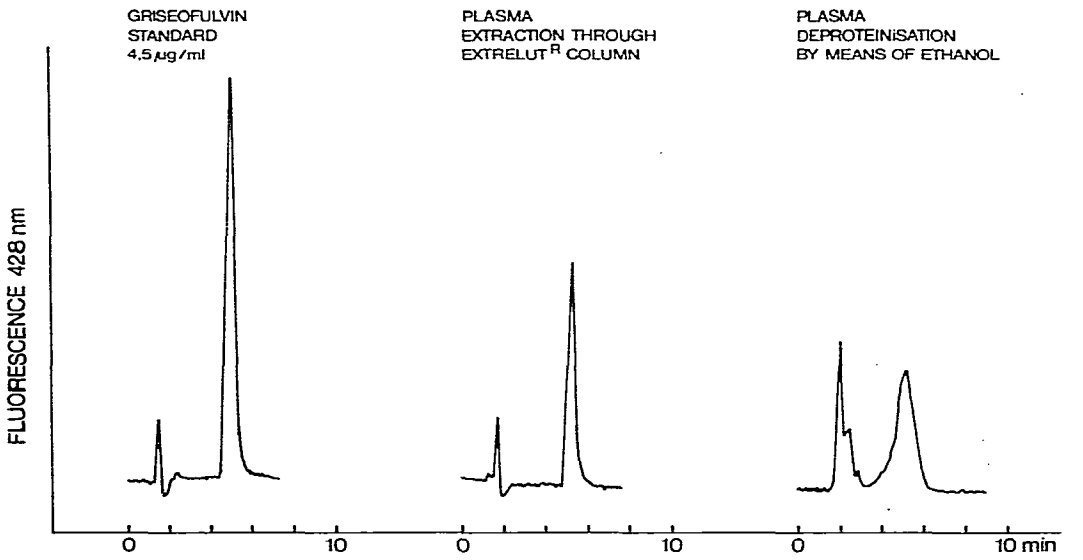

Fig. 2. HPLC chromatograms.

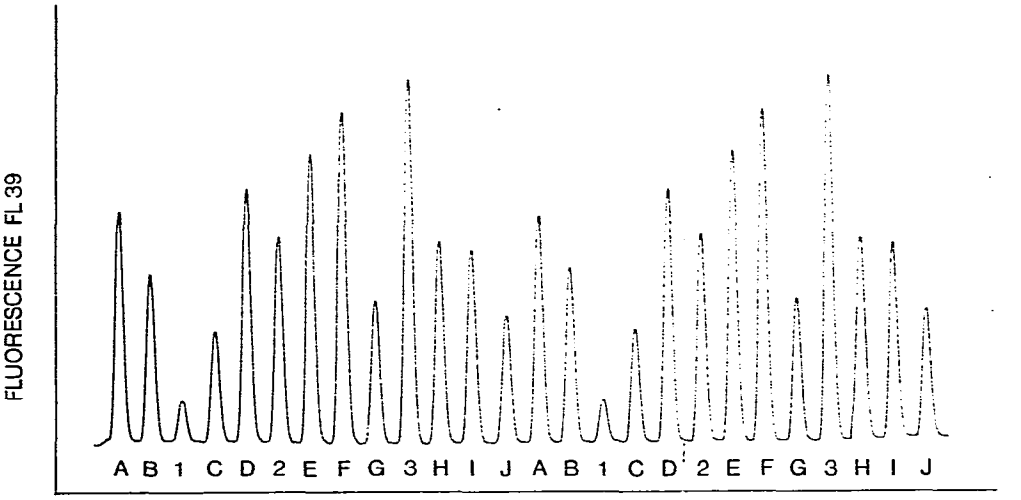

Fig. 3. HPTLC chromatogram.

DISCUSSION

The Extrelut^{R} extraction method for griseofulvin allows high recoveries over a wide range of concentrations, offers a sensible simplification in comparison to the conventional extraction, especially in serial analyses, and excludes faults to a large extent.

The advantage of HPLC is its high sensitivity, which could be enhanced by injecting larger volumes of the sample. The fact that the use of the cut-off filter with the Perkin-Elmer 204 yields a higher detection limit than with the monochromatic emission measurement, is due to the higher base-line noise in the cut-off filter measurement. The extraction of griseofulvin from plasma

is absolutely necessary and cannot be avoided by deproteinisation, as shown by the broader peak in Fig. 2.

The advantage of HPTLC is its simplicity, especially because no sample preparation is necessary. For this reason direct determination of griseofulvin in plasma samples is possible for the first time. The solvent causes deproteinisation at the site of application on the plate. As this precipitated protein causes enlarged spots after chromatography, dilution of the sample solution is necessary to reduce the amount of protein. It was of advantage to dilute with a solution of 2,5-dimethylbenzosulphonic acid, as in this way protein opacities of the plasma after a longer storage time are avoided. The speed of separation and measurement is remarkable; the measurement is executed at right-angles to the direction of chromatography (see Fig. 3). In contrast to the report of Fischer and Riegelman [7], the fluorescence signals are constant immediately. The only disadvantage of the HPTLC method is that with fluorescence excitation by an Hg lamp, the detection limit for griseofulvin is 0.15 μg/ml, and the useful measurement range begins at 0.5 μg/ml sample, that is at 1 μg griseofulvin per ml plasma. Using micro-optics for the Zeiss scanner this shortcoming can be overcome to a certain extent. There is the possibility to extract the sample in an Extrelut^{R} column, evaporate the ether and dissolve the residue in 0.5 ml of 50% methanol. This additional procedure augments the sensitivity four times.

As Table I shows, the HPTLC method requires only one sixth of the time necessary for the HPLC method, resulting in a cost ratio of 1:10. With an increase in sample numbers the preparation time decreases correspondingly. For both methods the ratio of manipulation is similar.

Consequently, for plasma containing more than 1 μg griseofulvin per ml, the HPTLC method should be preferred; for a lower griseofulvin content the HPLC method must be applied. This work shows how much the cost of analyses can be influenced when, according to the nature of the problem, one can choose among several chromatographic methods.

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