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DETERMINATION OF THALIDOMIDE AND ITS MAJOR METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and sensitive isocratic high-performance liquid chromatography assay for the simultaneous and quantitative determination of thalidomide (α-phthalimidoglutarimide) and its major metabolites from human serum has been developed. The parent compound and the metabolites can be efficiently separated by reversed-phase chromatography using tetramethylammonium bromide as an ion-pair-forming reagent.

INTRODUCTION

The renewed interest that thalidomide (α-phthalimidoglutarimide) presently attracts as an anti-inflammatory agent and as the drug of choice for the treatment of the lepra reaction (erythema nodosum leprosum, ENL), as well as of various other diseases of the skin and the mucous membranes [1], requires the development of a rapid and sensitive quantitative assay for both the parent compound and its metabolites (Fig. 1) in biological material, such as blood serum or tissue samples. Several analytical procedures for the detection of these compounds by colour reactions [2], UV spectrometry [3], paper chromatography [4–6], gas chromatography [7] and thin-layer chromatography [8] have been described. However, these methods are either rather inefficient and time-consuming or only suitable for qualitative determination.

In this paper, we report the separation and quantification of compounds I–IX after extraction from biological material by high-performance liquid chromatography (HPLC). The remaining compounds X–XIII, glutamic acid and the products derived therefrom, have been omitted, because they are either physiological substances undergoing rapid endogenous turnover or, in the case of compound

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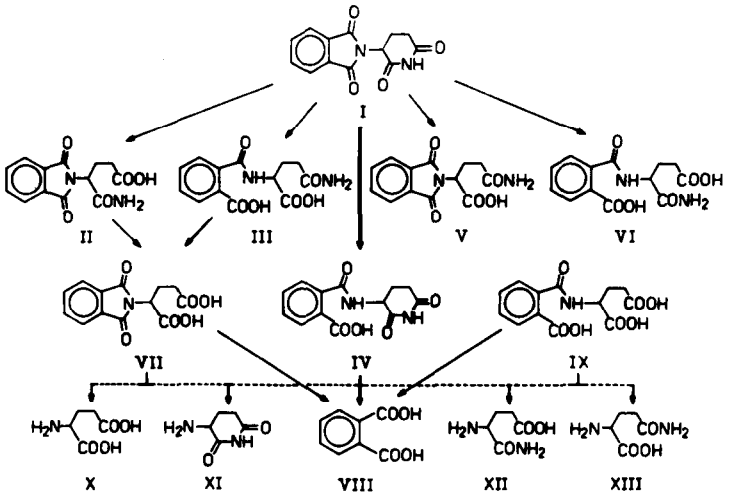


Fig. 1. Scheme of the hydrolysis of thalidomide.

XI, unlikely to occur among the natural metabolites of I under physiological conditions.

Thalidomide (I) is almost insoluble in water and only fairly soluble in most of the common organic solvents. The most appropriate media for the extraction and chromatography are dimethylformamide (DMF), tetrahydrofuran (THF), acetonitrile and *n*-propanol. The polar metabolites with free carboxylic groups (II–IX), however, are much more soluble in aqueous buffered systems of pH ca. 7, but decompose at increasing rates in solutions with higher pH values. In alkaline media, thalidomide is hydrolysed into several compounds (II–IX) which are identical with the main metabolites (IV, VII, VIII, IX) found in the human and animal organism and excreta, respectively [9,10].

EXPERIMENTAL

Chemicals

The compounds under test (I–IX) were synthesized by known methods [11]. An authentic sample of thalidomide was kindly supplied by Dr. E. Frankus from Chemie Grünenthal (Aachen, F.R.G.). All other chemicals were purchased from commercial sources. Water redistilled from a glass apparatus was used for all investigations.

Extraction from blood serum

Blood serum (1.5 ml, centrifuged at 2500 *g*) was placed on an extraction cartouche RP-18 (J.T. Baker, Gross-Gerau, F.R.G.) with 0.5 cm^3 stationary phase, wetted with 0.5 ml of saline (pH 7.4, sodium carbonate), washed with 0.1 *M* hydrochloric acid (0.5 ml) and eluted in several steps with various solvents (0.1 ml each). This procedure has the advantage of concentrating the samples in case the volume of the eluent be smaller than the sample volume. Then 20 μl of the

TABLE I

HPLC SEPARATION OF THALIDOMIDE AND ITS AROMATIC METABOLITES II-IX WITH ION-PAIR FORMATION (SYSTEM 1)

Compound No. (Fig. 2)	t_R (min)	k	α	R_s
IV	3.0	0.7		
			1.33	0.7
III	4.4	1.3		
			1.50	1.9
II	5.8	2.4		
			1.21	1.2
V, VI	7.0	3.1		
			1.21	1.3
VIII	8.6	4.0		
			1.14	0.9
VII	9.6	4.7		
			1.13	0.8
IX	11.0	5.4		
			1.17	3.9
I	18.7	9.9		

extract are placed onto the column and chromatographed with the mobile phase of either system 1 or 2, respectively (see below).

High-performance liquid chromatography

The HPLC instrumentation consisted of a Kontron Model 414 pump, an MSI 660T autosampler unit with a 20-μl loop, a 830 column oven, a Uvicon 740 LC absorbance detector, wavelength 254 or 290 nm (all from Kontron, Vienna, Austria) and a Shimadzu CR 1B integrator (Beckman, Vienna, Austria).

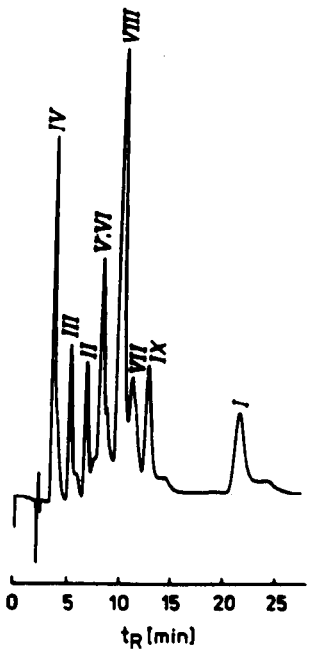
System 1 consisted of a Bischoff precolumn packed with 10-μm LiChrosorb RP-8, 10×4.6 mm I.D., and an analytical column packed with 5-μm LiChrosorb RP-8, 125×4.6 mm I.D.; these were connected by a cartridge system without any dead volume.

System 2 was a Spherisorb S5 C_{18} analytical column (12.6×0.46 cm I.D., Kontron).

The solvents used were HPLC-grade methanol and *n*-propanol, respectively (LiChrosolv, Merck, Darmstadt, F.R.G.), and reagent-grade DMF (Merck). The reagents used were tetramethylammonium bromide (TMAB) as ion-pair-forming agent, ethylene dinitrilotriacetic acid disodium salt as complexing agent and sulphuric acid as acidifier for the mobile phase; all reagents were of analytical grade (Merck).

RESULTS AND DISCUSSION

The chromatographic separation of the aromatic metabolites of thalidomide is difficult and time-consuming, because the compounds are structurally closely



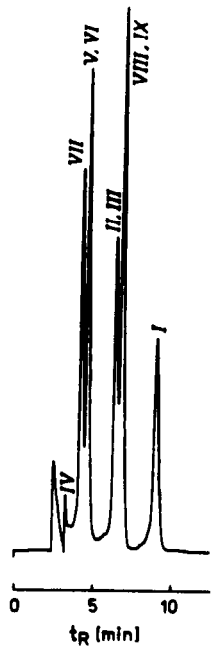


Fig. 2. HPLC separation of thalidomide and its aromatic metabolites II–IX with system 1. Eluent, water–methanol–*n*-propanol (180:30:20, v/v/v) plus 150 mg of sodium EDTA and 230 mg of TMAB; flow-rate, 0.9 ml/min; detection, UV at 254 nm and 0.02 a.u.f.s.; temperature, 30°C.

Fig. 3. HPLC separation of thalidomide and its aromatic metabolites II–IX with system 2. Eluent, water–acetonitrile (80:20, v/v) plus 120 mg of *n*-heptanesulphonic acid per ml and 150 µl of 30% hydrochloric acid per 100 ml; flow-rate, 1.5 ml/min; detection, UV at 290 nm and 0.02 a.u.f.s.; temperature, 30°C.

related to each other. However, complete separation was achieved through ion-pair formation with TMAB (Table I). Under these conditions (system 1), the total run requires ca. 25 min (Fig. 2).

For pharmacokinetic and in vitro stability determination studies, a second arrangement (system 2) was set up, without addition of the ion-pair-forming agent. The chromatographic resolution with system 2 is somewhat lower than that with system 1 (Table II). However, it offers the advantage of a much shorter total analysis time (Fig. 3).

When ion-pair formation is employed, the compounds are eluted in a different order compared with normal reversed-phase chromatography (Fig. 2). The only exception is the parent compound (I), which shows the highest k value in both systems; this is because it has no free carboxyl group and is thus not accessible to ion-pair formation.

The lower peak resolution efficiency of system 2 does not interfere with the application to in vitro studies, since the fraction of metabolites under one single peak is very low (1–3%). It can be neglected in comparison with the amounts of the main metabolites.

It is rather difficult to separate the isomeric compounds II and III for phar-

TABLE II

HPLC SEPARATION OF THALIDOMIDE AND ITS AROMATIC METABOLITES II–IX ON A REVERSED-PHASE COLUMN (SYSTEM 2)

Compound No. (Fig. 3)	t_R (min)	k	α	R_s
Injection	2.5	0		
			1.3	0.9
IV	3.2	0.3		
			2.7	1.8
VII	4.2	0.8		
			1.1	0.5
V, VI	4.6	0.9		
			1.8	1.8
II, III	6.3	1.6		
			1.1	0.4
VIII, IX	6.7	1.8		
			1.5	2.1
I	8.9	2.7		

macokinetic and biopharmaceutic studies under rational HPLC conditions. With N-cetyl-N,N,N-trimethylammonium bromide as ion-pair-forming agent, the time needed for complete separation is ca. 60 min, and even then the resolution is not of the best. Furthermore, the metabolites V and VI decompose quickly, even in chilled solutions ($-4°C$). Since V and VI barely contribute to the metabolic pattern, one can just dispense with their strict separation.

The exactness of the separation and the purity of a particular peak can be demonstrated through the correlation of peak width at half height versus the capacity factor. Overlapping is indicated already by an extreme breadth of a peak. In such cases, the correlation (Fig. 4) would lead to considerable deviation from the straight line of the respective point.

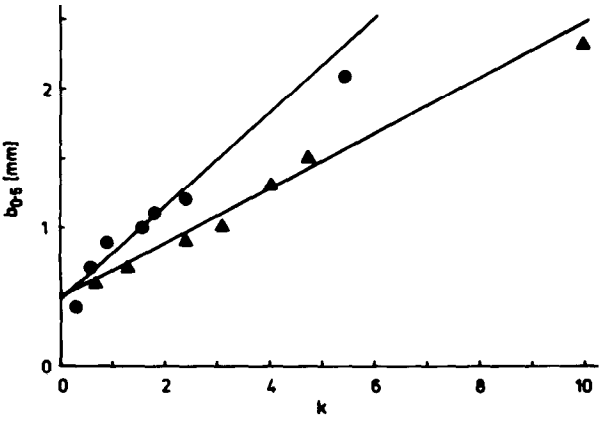


Fig. 4. Correlation of peak width at half height ($b_{0.5}$) versus capacity factor (k) for system 1 (●) and system 2 (▲).

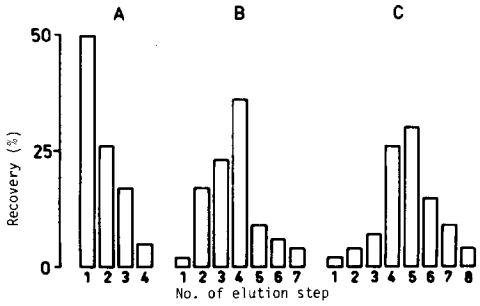


Fig. 5. Extraction profile for the isolation of thalidomide from human blood serum with DMF (A), DMF–methanol (B) and acetonitrile–methanol (C). Each bar represents the amount of thalidomide (%) that is released from the cartouche with 100 μl.

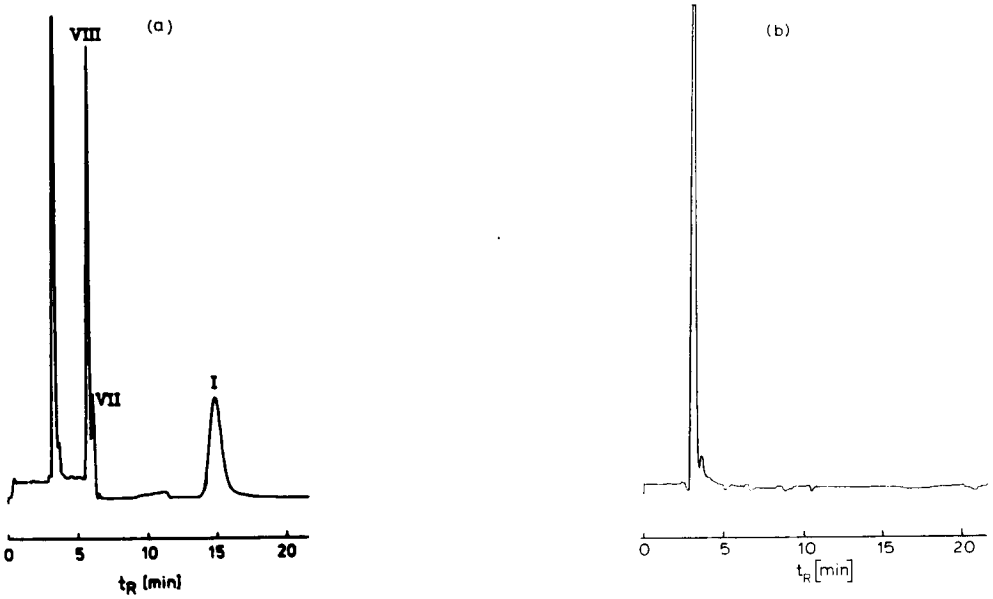


Fig. 6. (a) Typical chromatogram of thalidomide (I) and phthalic acid (VIII) after extraction from human blood plasma. Solvent as in Fig. 3; temperature, 50°C. (b) Plasma blank; HPLC conditions as in Fig. 3.

The extraction of thalidomide from blood serum was accomplished by means of Baker cartouches (see Experimental). The elution of the compound from the cartouche has been optimized using a variety of solvents: (a) DMF; (b) DMF–methanol (1:1, v/v); (c) acetonitrile–methanol (2:1, v/v).

The elution was performed in separate steps with 100-μl volumes of eluent in each step. In order to visualize the progress of the recovery of I, a chromatogram was produced after every step and the amounts obtained were plotted as a bar diagram (yield versus elution step) (Fig. 5). As can clearly be seen from Fig. 5, the bar diagram A represents the optimum elution profile (ca. 95% of the total), since after the fourth step nothing else was recovered. With this extraction technique, it was possible to eliminate the interfering plasma components by washing

them out with 0.1 *M* hydrochloric acid. No further interference of plasma constituents with substance peaks was found thereafter (Fig. 6).

This procedure was used to set up a calibration curve for the determination of I in human blood serum. The curve is linear in the range 1–100 μg/ml ($y=23.289x-25.271$; $r=0.998$). Actually, the parent drug (I) is the only compound of interest in pharmacological and pharmacokinetic investigations: the metabolites play only a minor role in such studies. In our experiment, the parent drug and metabolite VIII were found in every sample, whereas VII and IX were always present in lower concentrations than the former compounds, since they are rapidly hydrolysed (Fig. 6). The coefficient of variation from ten to twenty determinations, carried out on the same day, was between 2.3 and 7.4% for the concentration range 1–20 μg/ml; it was between 4.0 and 6.9% when estimated every other day within a period of three weeks (frozen samples, five determinations per day).

The prime advantage of the detection at 290 nm of I and its metabolites is an increase in the selectivity of the chromatographic separation. The loss of sensitivity during the determination at this wavelength (it counts for only about two thirds of that when measured at 254 nm) is nevertheless acceptable, since I is administered at relatively high doses that also result in high blood levels. Furthermore, the loss of sensitivity is more than compensated by the sample concentration during the extraction procedure (decrease of sample volume from 1.5 to 0.4 ml). The lower limit of detection of I is 0.5–1 μg/ml, when a 20-μl loop is used ($n=6$, signal-to-noise ratio >5).

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