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DETERMINATION OF THREE MAIN ANTILEPROSY DRUGS AND THEIR MAIN METABOLITES IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The simultaneous analysis of main antileprosy drugs such as 4,4′-diaminodiphenyl sulfone (DDS), clofazimine, rifampicin and their main metabolites in serum was examined by high-performance liquid chromatography using a μBondapak C_{18} column. When the drugs dissoluted from serum were developed by tetrahydrofuran—0.5% acetic acid (40:60), clofazimine and rifampicins could be analyzed separately. Apart from the mutual separation of water-soluble conjugates of DDS, the individual analysis of DDS, its main liposoluble metabolite and a few related sulfone compounds is possible when the drugs are first developed by acetonitrile—water (20:80). By the use of tetrahydrofuran—water (50:50) containing PIC B-5, the rapid measurement of clofazimine isolated from the other compounds is also possible.

INTRODUCTION

The main antileprosy drugs presently used are 4,4′-diaminodiphenyl sulfone (DDS) [1], 3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylamino)-phenazine (clofazimine) [2] and rifampicin (RFP) [3], of which the former two have pharmacodynamic activities [4]. Moreover, we have found that DDS and clofazimine have depressive and potentiative effects, respectively, on

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cell-mediated immunity of thymectomized guinea pigs when examined by a rabbit red blood cell rosette formation method [5].

Up to now, Gordon and Peters [6] and Burchfield et al. [7] have reported, respectively, fluoriphotometric and gas chromatographic analysis of DDS and its acetylated metabolites. Both of these highly sensitive methods brought a significant advancement in pharmacokinetic and clinical studies on leprosy. A favorable monitoring method for detecting excreted urinary DDS from patients was also reported [8]. On the other hand, Dill and Glazko [9] reported a fluoriphotometric method for analyzing clofazimine using titanous chloride and sulfuric acid. Vlasáková et al. [10] reported the separation of RFP

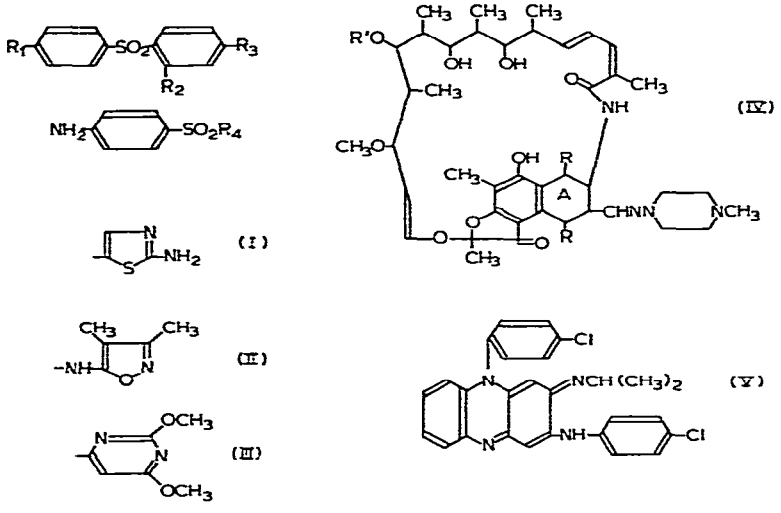

Fig. 1. Structural formulae of antileprosy drugs and related substances.

	R_1	R_2	R_3
DDS	NH_2	H	NH_2
MADDS	NH_2	H	$NHCOCH_3$
DDSG	NH_2	H	$NHC_6H_8O_6Na$
DDSS	NH_2	H	$NHSO_3K$
s-DDS	NH_2	SO_2NH_2	NH_2
Proethyl	NH_2	H	$NHCH_2CH_2OH$

	R_4
Promizole	I
Sulfisoxazole	II
Sulfadimethoxine	III

IV	R	R'	A
RFP	OH	CH_3CO	naphthol
DARFP	OH	OH	naphthol
RFPQ	O	O	naphthoquinone

V: Clofazimine

and its hydrogenated substances by high-performance liquid chromatography (HPLC). However, no complete chromatographic separation of all of these antileprosy drugs and their metabolites has yet been achieved.

In the meantime, since the occurrence of patients resistant to DDS therapy has gradually become a serious problem in the field of leprosy where DDS has long been used as the single and economical drug especially established for antileprosy chemotherapy, attention has been given to a combined therapy using DDS with the other two drugs.

In order to facilitate the analysis of serum specimens of patients under the combined therapy, we have examined the simultaneous analysis of the drugs in a pooled guinea pig serum by HPLC.

EXPERIMENTAL

Analytical instruments

A liquid chromatograph system (Waters Assoc., Milford, MA, U.S.A.) consisted of a high-pressure pump (Model 6000A) and a universal injector (Model U6K) equipped with a Jasco Uvidec 100-II UV spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan) was used. A recorder (Model VP-6621-A, Matsushita Communication Ind. Co., Yokohama, Japan) was linked to the detector. The attenuations of the UV spectrophotometric detector were between 0.005 (minimum range) and 0.04 depending on drug concentration. The column used was μBondapak C_{18} (300 mm × 3.9 mm I.D., Waters Assoc.).

For UV spectrophotometry, an Hitachi recording spectrophotometer, Model EPS-3T (Hitachi-Nissei, Tokyo, Japan) was used.

Mobile phases

The compositions of the mobile phases used were: system A, acetonitrile—water (20:80); system B, tetrahydrofuran (THF)—0.5% acetic acid (40:60); and system C, THF—water containing PIC B-5 (50:50). The reagent PIC B-5 (Waters Assoc.) contains 1-pentanesulfonic acid and glacial acetic acid, the concentration of the acetic acid being a trade secret. The concentration of the 1-pentanesulfonic acid in water was 0.005 *M* , thus its final concentration in system C was 0.0025 *M*. The flow-rates for systems A, B and C were 2.0 ml/min (70 kg/cm^2), 1.5 ml/min (112 kg/cm^2) and 1.5 ml/min (116 kg/cm^2), respectively. All the organic solvents in systems A—C were filtered through a Teflon filter (FHLP04700, pore size 0.5 μm, Millipore). The re-distilled water was filtered through a cellulose ester filter (GSWP04700, pore size 0.22 μm, Millipore). After mixing an organic solvent with an aqueous layer inside a graduated glass cylinder, which was well washed with the filtered water, every mobile phase was degassed in a specially cleansed glass bottle using a sonicator (Ultrasonic Cleaner B-220, Branson and Smithkline) for at least 20 min before use.

Switching of mobile phase

Since RFPs and clofazimine could first be separated by development with system B, whereas sulfones were separable with system A, in order to achieve simultaneous analysis of all the drugs, the switching of the mobile phases from

system A to B or from B to A was performed. For switching from A to B, system B was run into the analytical system after analysis of sulfones with system A. However, for switching from B to A, system B was first removed by pumping methanol—water (50:50) through the system at 1.0 ml/min for at least 20 min before starting column conditioning with system A.

Chemicals

All of the following drugs were kindly supplied by the following companies. DDS, 2-amino-5-sulfanilylthiazole (promizole, PZ) and 4-hydroxyethyl DDS (proethyl, PE), Yoshitomi, Fukuoka, Japan; DDS 2-sulfonamide (s-DDS) [11], Tanabe, Saitama, Japan; sulfisoxazole, Yamanouchi, Tokyo, Japan; sulfadimethoxine, Chugai, Tokyo, Japan; clofazimine, Ciba-Geigy, Basle, Switzerland; purified standards of RFP and its analogues, 25-desacetyl rifampicin (DARFP) and rifampicin 1,4-quinone (RFPQ), Daiichi, Tokyo, Japan under licence from Lepetit, Milan, Italy. DDS 4-mono-N-acetylate [12] (MADDS) was synthesized in this laboratory.

The other compounds used in search of a suitable internal standard for the RFPs and clofazimine were kindly supplied by Nihon Waters, Tokyo, Japan.

All of the solvents used for HPLC and UV spectrophotometry were those specially purified for HPLC and obtained from Tokyo Kasei Kogyo, Tokyo, Japan, and Wako Pure Chemical, Osaka, Japan. The solvents and chemicals used for extraction or the other procedures were of guaranteed reagent grade or special reagent grade of the two respective companies.

Standard solutions

All the substances were dissolved in suitable solvents usually at a concentration of 2 μmol/ml. Sulfone compounds other than s-DDS were dissolved in methanol. s-DDS and sulfonamides excluding sulfisoxazole were dissolved once in a small volume of 0.1 *M* NaOH and diluted with methanol to a final alkali concentration below 0.01 *M*. But when s-DDS was used as an internal standard it was dissolved in the solvent system distilled water—chloroform—dimethylformamide (DMF) (1:1:5) which was used for the dissolution of drugs in serum and named SEx. Sulfisoxazole was dissolved in water, clofazimine was dissolved in chloroform. The stock solutions of these drugs were kept in an Ultra-low Revco Freezer and used within 2 weeks after preparation. All RFPs are dissoluble in chloroform or isoamyl alcohol. However, since they are unstable in both solvents when examined spectrophotometrically [13], in cases when they were analysed individually they were dissolved in DMF just before use because DMF strongly stabilizes RFPs [14]. However, for determination of the redissolution ratios from serum of RFPs and for analysis of their serum samples by HPLC, they were once dissolved in chloroform and the chloroform was immediately evaporated to 70—1400 nmol and 42—420 nmol, respectively.

UV spectrophotometry

The stock solutions (2 μmol/ml) were diluted to 10 nmol/ml (clofazimine) or 20 nmol/ml (sulfone compounds, sulfonamides and RFPs) with the developing solvents to be used for HPLC and immediately analyzed. However, the

substances selected as standards were analyzed at the final concentrations of 5—50 nmol/ml.

Selection of standards

The standards were selected based on their retention times and UV absorbances which were analyzed spectrophotometrically. On the basis of these results, we selected s-DDS for analysis of sulfone compounds and phenylthiohydantoin(ε-phenylthiocarbamyl)-lysine (PEPL) for analysis of RFPs and clofazimine (Table I). All of the reagents shown in Table I were of guaranteed reagent grade of Tokyo Kasei Kogyo.

Sera

In order to use guinea pigs as an animal model for examining the influences of antileprosy drugs on cell-mediated immunity, which is supposed to be closely connected with the main cause of relapsed or persistently positive patients [5], we preferentially used a guinea pig serum pooled after collection from hearts of more than 20 healthy animals (Hartley, 600—750 g). Blood

TABLE I

SELECTION OF STANDARDS BASED ON THEIR UV ABSORBANCE (MEASURED BY SPECTROPHOTOMETRY) IN SYSTEM A OR B, AND THEIR RETENTION TIMES WHEN DEVELOPED BY HPLC

Substance	Spectrophotometry		HPLC	
	Peak (nm)*	Molecular extinction × 10^{-4}	t_R (min)	Developing solvent**
Sulfisoxazole	255	2.27	2.5	System A
Promizole (PZ)	269, 334	2.09, 3.41	5.4	System A
s-DDS	262, 297	1.84, 2.45	6.7	System A
DDS	261, 296	1.70, 2.86	7.5	System A
Proethyl (PE)	266, 306	1.80, 3.29	7.8	System A
MADDS	259, 295	2.01, 2.51	9.9	System A
Sulfadimethoxine	272	2.53	12.6	System A
DARFP	242, 256, 338	3.38, 3.30, 2.60	6.9	System B
PTH-phenylalanine***	271	1.01	8.2	System B
RFP	242, 256, 337	4.45, 3.95, 3.13	8.2	System B
RFPQ	242, 278, 337	2.82, 2.61, 1.75	9.4	System B
PTH(ε-PTC)-lysine§ (PEPL)	270	2.93	12.6	System B
Acenaphthene	234, 283, 292, 303, 309, 323	2.22, 0.88, 0.99, 0.62, 0.44, 0.26	18.8	System B
Clofazimine	287	8.82	19.1	System B
Fluoranthene§§	216, 239, 279, 289	4.35, 5.55, 2.35, 4.40	20.1	System B

*UV absorbance was measured by an Hitachi Recording Spectrophotometer, Model EPS-3T.
**System A: acetonitrile—water (20:80), flow-rate 2.0 ml/min, pressure 70 kg/cm^2. System B: tetrahydrofuran—0.5% acetic acid (40:60), flow-rate 1.5 ml/min, pressure 112 kg/cm^2.
***PTH = phenylthiohydantoin.
§PTC = phenylthiocarbamyl.
§§Only main peaks recorded.

TABLE II

DISSOLUTION RATIO FROM SERUM SPECIMENS OF EACH DRUG (AVERAGED ON THE BASIS OF THOSE AT VARIOUS CONCENTRATIONS) AND THE LINEARITY BY OPTICAL DENSITY OF VARIOUS CONCENTRATIONS OF DRUG IN THE REDISSOLUTE

One millilitre each of various concentrations of each drug (70, 140, 210, 350, 700 or 1400 nmol/ml) prepared by diluting the stock solution (2 μmol/ml) was separately dried in vacuo. The residues were dissolved by 7 ml of water—chloroform—DMF (1:1:5) (SEx) as the comparative controls for 100% redissolution. In addition, in order to examine the sufficient redissolution from serum specimens, 1 ml of bovine serum, 1 g of $(NH_4)_2SO_4$, 1 ml of chloroform and 5 ml of DMF were stepwisely added to the dried drug in various amounts (70, 140, 210, 350, 700 or 1400 nmol) under stirring. After centrifugation followed by filtration, the optical densities of the supernatants were measured using an Hitachi Recording Spectrophotometer, Model EPS-3T, and compared with those of comparative controls (SEx solutions). The composition of the final supernatants obtained from serum specimens is an aqueous layer of serum—chloroform—DMF (1:1:5), which is named SExS in this paper, in addition to various amounts of drug. Always, three specimens were prepared at each drug concentration. All of the optical densities (comparative controls, n = 18) and of redissolution ratios (serum specimens, n = 18) at the final concentrations of 10—200 nmol/ml were found to be within ± 10% error, and 1—3 values at each concentration were used for determining the redissolution ratio averaged and statistically examined by C.V.

Substance	nm	Y (optical density) = aX (nmol) + b						Averaged dissolution ratio (%)	C.V. (%)
		Control			Redissolute				
		a*	b	n**	a'	b'	n		
DDS	310	0.0325	−0.0486	16	0.0314	0.0114	12	102.9	5.11
MADDS	303	0.0263	0.0073	15	0.0254	0.0028	12	100.3	1.40
PZ	310	0.0309	−0.0090	16	0.0303	−0.0590	13	101.9	2.50
s-DDS	303	0.0237	−0.0318	14	***	—	—	—	—
Clofazimine	289	0.0849	−0.0831	13	0.0865	−0.0472	12	99.8	4.33
Clofazimine	452	0.0592	−0.0026	16	0.0599	−0.0266	14	99.6	5.32
RFP	342	0.0273	0.0226	13	0.0270	0.0315	13	100.6	2.19
DARFP	342	0.0271	−0.0023	14	0.0275	0.0053	10	100.0	1.74
RFPQ	263	0.0264	−0.0262	13	0.0253	−0.0365	10	101.5	4.87
RFPQ	330	0.0125	−0.0370	15	0.0128	−0.0226	12	104.8	4.11
PEPL	272	0.0293	0.0203	16	***	—	—	—	—

*Linear coefficients determined by the least squares method.
**Number of specimens used for determining linear coefficients.
***Since both s-DDS and PEPL used as standard materials were added to the supernatants after redissolution, their redissolution ratios are not given.

samples were taken whenever needed and preserved at −80°C as 5—10-ml aliquots in sterilized disposable centrifuge tubes (Polyspitz-S, Nissui). However, for determination of redissolution ratios of drugs, in order to decrease the frequency of sampling, bovine serum (Japan Biotest Institute) was used.

Determination of redissolution ratios of drugs

Starting from each stock solution, 70, 140, 210, 350, 700 or 1400 nmol of each drug were dried in vacuo below 40°C in a glass test tube (100 mm long, 15 mm I.D.) by the use of a shaking evaporator, Vapour-Mix (Tokyo Rikakikai). One millilitre of bovine serum was added and stirred for 20 sec by a Micro Thermo-Mixer, Model TM-101 (Tokyo Thermonics) to take up the drug in the serum. Then 1 ml of chloroform and 1 g of $(NH_4)_2SO_4$ fine crystals were added and again stirred for 20 sec. To the resultant homogenate, 5 ml of DMF were added and stirred further for 1 min. The tube was centrifuged at 4000 *g* for 10 min at 0°C on a Marusan Model 50M (-L) centrifuge (Sakuma Seisakusho). The supernatant was filtered once through a filter paper (Toyo Roshi No. 7, dial 9 cm, Toyo Roshi). In addition, 8 ml of the bovine serum were extracted with increased amounts of redissolution reagents. The final filtrate, with a composition equal to SEx except that it contains protein-free supernatant of bovine serum instead of water as in SEx, was named SExS. It was used for diluting the portions of redissolutes where the optical densities were above 1.0 due to the high drug concentrations.

As the comparative control, the drug solutions of 10, 20, 30, 50, 100 and 200 nmol/ml of SEx were used as standards for 100% extraction.

In each case three specimens were prepared at each drug concentration. The average redissolution ratio of each drug represented by the coefficient of variation (C.V.) was elicited on the basis of 1—3 ratios at each concentration. The linearity of response of each drug was examined by the least squares method. This deproteinated redissolution could minimize the volume of denaturated protein block-precipitated after centrifugation. Without the addition of chloroform, a slight turbidity was occasionally observed in the supernatant. The results are shown in Table II.

Redissolution of drugs from serum for HPLC analysis

Initially, the following solutions were prepared. (I) Sulfone compounds (each 100 μmol) excluding s-DDS and 100 μmol of clofazimine together dissolved in 50 ml of methanol. (II) RFP and DARFP (each 64 μmol) dissolved in 8 ml of chloroform. (III) RFPQ (64 μmol) dissolved in 8 ml of chloroform. (IV) s-DDS (40 μmol) dissolved in 200 ml of SEx. (V) PEPL (40 μmol) dissolved in 200 ml of SEx. By diluting solution I with methanol, 10-ml solutions of drugs containing 200, 500, 1000 or 2000 nmol/ml were prepared; 0.21-ml aliquots of each were immediately dried in vacuo in 20 glass test tubes. These were preserved at −20°C until use. Solution II was immediately washed by stirring for 30 sec with 0.5 ml of 1% ascorbic acid aqueous solution. It was washed further twice with 0.5 ml each time of distilled water. The chloroform layer was diluted with chloroform to 0.8, 2.0 or 4.0 μmol/ml. An aliquot (0.21 ml) of each of the original solution or dilutions were dried in vacuo below 40°C in 12—15 glass test tubes. The residues contain 168, 420, 840 or 1680

nmol of RFP and DARFP. They were preserved at −80°C until use. Just before use, 1 ml of chloroform was added and an aliquot (0.25 ml) of the resultant solution was added to the residue dried from solution I. The mixture was immediately dried in vacuo below 40°C. Solution III was diluted with chloroform as in the case of solution II but without washing the chloroform layer. It was layered on to the dried mixture of solutions I and II and immediately dried again in vacuo below 40°C. Thus, the final dried residue contains 42, 105, 210 or 420 nmol of PZ, DDS, MADDS, RFPs and clofazimine.

To the dried drug mixture, 0.3 ml of pooled guinea pig serum and 0.3 g of $(NH_4)_2SO_4$ were added. It was made to redissolute by the further addition of 0.3 ml of chloroform and 1.5 ml of DMF. After centrifugation at 4000 *g* for 10 min at 0°C, 1 ml of the supernatant whose drug concentration is to be 20, 50, 100 or 200 nmol/ml was diluted with 1 ml of solution IV or V, in which the concentration of s-DDS or PEPL is equal or twice that of the drugs, respectively. In order to estimate the practical minimum measurable quantity, in some cases the redissolute of the lowest final concentration (10 nmol/ml) was further diluted with SExS.

All of the redissolutes were filtered through a Teflon-type filter (FHLP01300, pore size 0.5 μm, Millipore) and analyzed by HPLC; the injection volume was 3—8 μl, a Hamilton micro-syringe, Microliter No. 820 being used.

RESULTS AND DISCUSSION

At the first step, the discovery of a suitable solvent in which are freely dissoluble all the sulfone drugs, clofazimine and RFPs in serum was requested.

Ellard and Gammon [15] and Peters and co-workers [16, 17] extracted DDS and MADDS from serum with ethyl acetate and from urine with dichloroethane at a strongly basic pH. Barry et al. [18] and other investigators [19, 20] used chloroform or acetic acid—benzene (or toluene) (1:3) for extracting clofazimine from biological materials. RFPs are extractable quantitatively with chloroform, isoamyl alcohol [21] or other solvent mixtures [22]. We also noticed that clofazimine and RFPs could be sufficiently extracted by deproteinative extraction with chloroform and ammonium sulfate even in final concentrations above 200 nmol/ml. However, since extraction at acidic or basic pH is undesirable for the unstable RFPs, we could not use chloroform as the extraction solvent because especially the extraction ratios of sulfones after agitation with chloroform at neutral pH were found to be incomplete above a final concentration of 50 nmol/ml. Although the method used in this report is not extraction but a mere redissolution, this method showed two advantages: (1) the minimization of denaturated protein block-precipitated after centrifugation of redissolute; and (2) the stabilization of RFPs by the effect of DMF.

The redissolute was first developed by system A which is the usual developing solvent system for analyzing sulfonamides on a μBondapak C_{18} column [23]. Various quantities between 5 and 500 pmol of liposoluble sulfone compounds were analyzed. The chromatograms at lower concentrations of drugs and the linearities examined by coefficients of variation are shown in Fig. 2 and Table III, respectively. The linearities by both peak height and peak area could clearly be found in the range of attenuator on the UV spectrophotometric detector linked to the outlet of the column from 0.005 (minimum

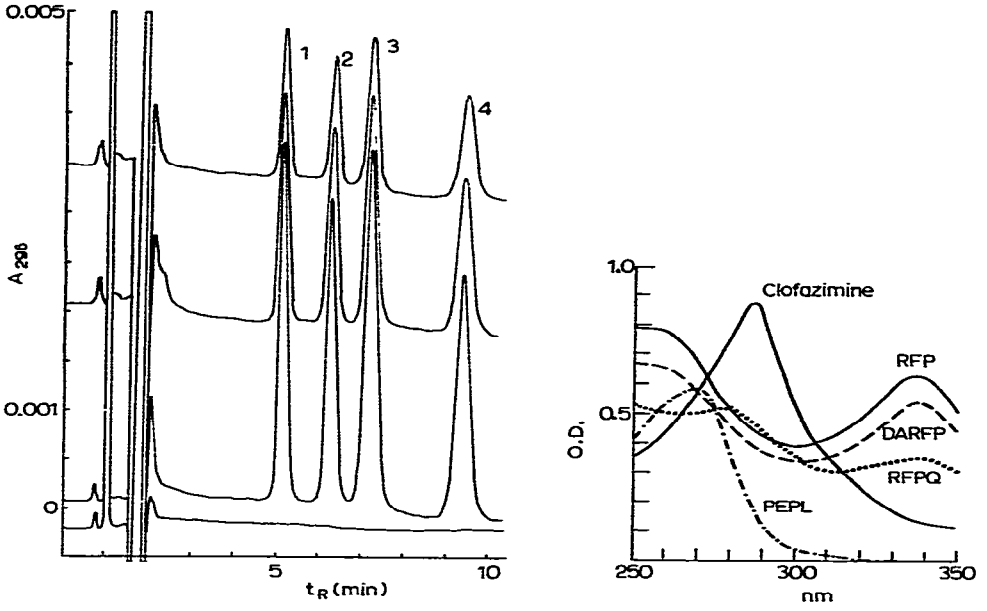

Fig. 2. Chromatograms of several sulfone compounds developed with system A and detected at 296 nm. Developing solvent A = acetonitrile—water (20:80); flow-rate 2.0 ml/min; pressure, 70 kg/cm²; temperature, 20 ± 2°C. Peaks: 1 = promizole (PZ); 2 = 4,4'-diaminodiphenyl sulfone 2'-sulfonamide (s-DDS); 3 = 4,4'-diaminodiphenyl sulfone (DDS); 4 = DDS 4-mono-N-acetylate (MADDS). The chromatograms from top to bottom correspond, respectively, to 30 pmol, 50 pmol, and 80 pmol of each drug. Range on attenuator: 0.005. Both of the two sharp large peaks detected at t_R 1.1 and 1.8 min are those of the basal medium [SExS, the aqueous layer of guinea pig serum—chloroform—DMF (1:1:5)]. By developing with system A, only the sulfone compounds eluted and all the RFPs, PEPL and clofazimine remained on the μBondapak C_{18} column.

Fig. 3. UV spectra of RFPs, clofazimine and PEPL (as the standard for HPLC analysis). Solvent system B. Concentrations of substances: clofazimine, 10 nmol/ml; the others, 20 nmol/ml. Spectrophotometer: Hitachi Recording Spectrophotometer, Model EPS-3T.

range) to 0.04. Based on a signal-to-noise ratio above 30, all of the practical minimum measurable quantities of DDS, MADDS and PZ were found to be 15 pmol when injection volumes of 3—8 μl of 2 nmol/ml of redissolute were analyzed. The averaged recovery ratios (peaks of redissolute/peaks of SEx solution, %) and C.V. (%) at low molecular concentrations (10 pmol per 5 μl and 16 pmol per 8 μl) were: PZ 102.4%, 3.05%, n = 3; DDS 99.0%, 5.09%, n = 3; MADDS 99.1%, 4.72%, n = 4.

When the redissolute was developed with system B, DDS, MADDS and PZ were eluted at the same time as a single peak. Then, the peaks of RFPs were detected in the order DARFP, RFP and RFPQ. The peaks of PEPL and clofazimine were found later than the elution of RFPs.

However, as the wavelengths of the main RFPs peaks are shorter or longer than those of clofazimine and PEPL, the selection of a suitable detection wavelength was requested.

As shown in Fig. 3, the molecular extinction of PEPL markedly decreased from the peak at 270 nm to a longer wavelength (29,300, 9500, 2520, 890 and 310 at 270, 287, 300, 310 and 320 nm, respectively); 2, 5 or 20 times higher concentrations of PEPL as standard than that of drugs were needed when analyzed by HPLC at 287, 300, and 310—320 nm, respectively. The

TABLE III

STATISTICAL RESULTS OF CHROMATOGRAMS OF SULFONE COMPOUNDS DEVELOPED WITH SYSTEM A AND DETECTED AT 296 nm

Substance	t_R (min)	Peak height* (= mm/nmol) (R: 1.0)	C.V. (%)	Peak height (= aX (pmol) + b)		Peak area** (mm^2/nmol) (R: 1.0)	C.V. (%)	Peak area (= $a'X$ (pmol) + b')		Pract. min.§ (pmol)
				a***	b***			a'	b'	
PZ	5.2	11.6	3.53	0.0107	0.0512	47.7	2.83	0.0448	0.0867	15
s-DDS§§	6.4	10.0	2.00	0.0098	0.0181	47.9	2.00	0.0460	0.1868	15
DDS	7.2	11.0	3.45	0.0105	0.0468	59.9	3.99	0.0577	0.1995	15
MADDS	9.4	8.2	1.83	0.0070	0.0268	59.1	3.37	0.0529	0.0156	15

*Each peak height (mm) was converted into that at 1 nmol and the range (R) on the attenuator at 1.0 after correction by that of s-DDS as the standard. Then all the peak heights were averaged and examined statistically by C.V.

**The peak areas (height × width at half height) were statistically treated in the same way as the peak heights.

***The linearity was examined by the least squares method. The linear coefficients (a and b for peak heights and a' and b' for peak areas) based on heights or areas of five adequate peaks (from 10 to 500 pmol) are recorded.

§ The practical minimum measurable quantity was estimated from chromatograms based on a signal-to-noise ratio above 30.

§§ The values of s-DDS peaks were based on those developed in SEx solutions alone because it is the standard for sulfone compounds.

sensitivity in peak detection of PEPL itself by HPLC analysis examined by minimum measurable quantity was also found to be lower when the solution or dissolute of PEPL which was dissolved in SEx [water—chloroform—DMF (1:1:5)] or in SExS [serum—chloroform—DMF (1:1:5) with added ammonium sulfate], respectively, was analyzed.

However, when all RFPs, clofazimine and PEPL were analyzed at 287 nm, which is the optical peak of clofazimine dissolved in system B, the peaks of RFPs, especially of RFPQ, were larger than those at 300 nm, due to the gradual increase in the UV absorbance of RFPs from 300 nm to 287 nm. For this reason, we concluded that analysis at 287 nm after mixing a two-times higher concentration of PEPL than that of the drugs would be the most rational method.

Various quantities of RFPs (10—500 pmol) and clofazimine (5—500 pmol) were analyzed. The representative chromatogram and the linearities examined at 287 nm are shown in Fig. 4 and Table IV, respectively. In Table IV, the practical minimum measurable quantities were estimated on the basis of a signal-to-noise ratio above 30. The averaged recovery ratio (%) and C.V. (%) at low molecular concentrations (10 pmol per 2 μl, 30 pmol per 6 μl, 50 pmol

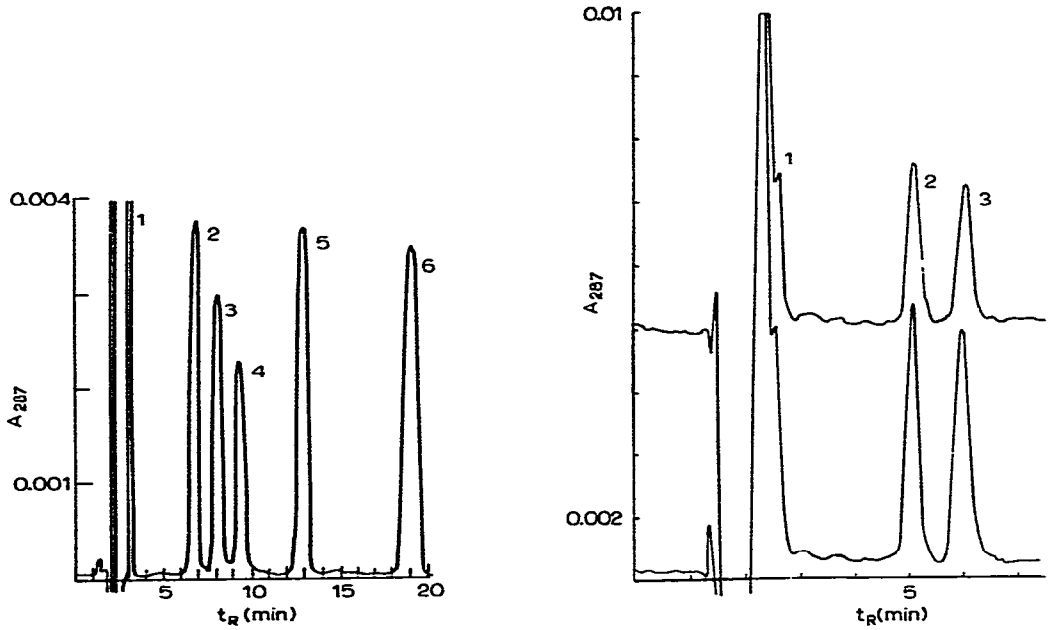

Fig. 4. Chromatograms of RFPs, clofazimine and PEPL (as the standard) developed with system B and detected at the optical peak of clofazimine (287 nm). Developing solvent B, THF—0.5% acetic acid (40:60); flow-rate, 1.5 ml/min; pressure, 112 kg/cm²; temperature, 20 ± 2°C. 1 = Peaks from mixture of DDS, MADDS and PZ; 2 = 25-desacetyl RFP (DARFP); 3 = RFP; 4 = RFP 1,4-quinone (RFPQ); 5 = PTH(ϵ-PTC)-lysine (PEPL); 6 = clofazimine. Concentrations: PEPL, 200 pmol; all of the others, 100 pmol. Range on attenuator, 0.01.

Fig. 5. Chromatograms of clofazimine isolated from RFPs when developed with system C. Developing solvent C, THF—water (50:50) containing PIC B-5; flow-rate, 1.5 ml/min; pressure, 116 kg/cm²; temperature, 20 ± 2°C. 1 = Peaks from mixture of solvent, sulfone compounds and RFPs; 2 = peaks of clofazimine, upper peak with 15 pmol, lower peak with 25 pmol; 3 = peaks of PEPL, upper peak with 60 pmol, lower peak with 100 pmol. Range on attenuator: 0.01.

TABLE IV

STATISTICAL RESULTS OF CHROMATOGRAMS OF RFPs AND CLOFAZIMINE DEVELOPED WITH SYSTEM B AND DETECTED AT 287 nm

The meanings of linear coefficients and the practical minimum measurable quantities, or the statistical method, are the same as those explained in the footnotes of Table III.

Substance	t_R (min)	Peak height (=mm/nmol) (R: 1.0)	C.V. (%)	Peak height (=aX (pmol) + b)		Peak area (=mm²/nmol) (R: 1.0)	C.V. (%)	Peak area (=$a'X$ (pmol) + b')		Pract. min. (pmol)
				a	b			a'	b'	
DARFP	6.5	7.1	2.54	0.0070	0.0019	57.1	3.71	0.0553	0.3007	40
RFP	7.8	5.4	5.56	0.0059	−0.1322	42.8	7.71	0.0521	−1.8824	40
RFPQ	9.2	4.3	6.51	0.0045	0.0833	59.6	4.40	0.0574	0.1135	50
PEPL	12.5	3.4	3.24	0.0034	−0.0357	32.9	3.25	0.0350	−0.6501	50
Clofazimine	18.9	6.3	4.60	0.0059	0.0701	104.3	2.36	0.1012	0.5444	15

TABLE V

STATISTICAL RESULTS OF CHROMATOGRAMS OF CLOFAZIMINE ISOLATED FROM RFPs WHEN DEVELOPED WITH SYSTEM C AND DETECTED AT 287 nm

The meanings of linear coefficients and the practical minimum measurable quantities, or the statistical method, are the same as those explained in the footnotes of Table III.

Substance	t_R (min)	Peak height (=mm/nmol) (R: 1.0)	C.V. (%)	Peak height (=aX (pmol) + b)		Peak area (=mm²/nmol) (R: 1.0)	C.V. (%)	Peak area (=$a'X$ (pmol) + b')		Pract. min. (pmol)
				a	b			a'	b'	
Clofazimine	5.1	42.8	11.42	0.0397	0.0745	190.9	2.34	0.1844	0.4005	5
PEPL	6.1	8.7	1.61	0.0083	0.1439	52.1	1.63	0.0503	0.7157	20

per 5 μl) were: DARFP 104.6%, 2.78%, $n = 3$; RFP 98.1%, 7.90%, $n = 3$; RFPQ 103.0%, 5.31%, $n = 3$; clofazimine 97.8%, 8.85%, $n = 4$.

Some other solvent systems were examined together. However, clofazimine could not be eluted with methanol systems containing 0.5% phosphoric acid, 1% acetic acid or PIC B-5, and the elution rates with solvent systems containing THF and various volumes of 0.5% phosphoric acid were too fast.

If the redissolute was developed with THF—water (50:50) containing 0.0025 *M* 1-pentanesulfonic acid and acetic acid as PIC B-5, the elution pattern was faster than that in development by system B. As the result, the rapid measurement of clofazimine separated from RFPs became possible. In this case, the peak of PEPL was found later than that of clofazimine. Representative chromatograms and the statistical results are shown in Fig. 5 and Table V, respectively. For analyzing clofazimine by development with system C, a final concentration of PEPL four times higher than that of clofazimine was used. The averaged recovery ratio (%) and C.V. (%) at low molecular concentrations (3 pmol per 3 μl, 5 pmol per 5 μl, 10 pmol per 5 μl, 16 pmol per 8 μl) was: clofazimine 97.8%, 6.68%, $n = 4$.

Insofar as the analysis could be finished within a day, each of the purified RFP standards showed a fine single peak when dissolved in the DMF or SEx system and preserved in an ice bath. At the same time, even if they were kept under these conditions before analysis, a non-negligible quantity of RFPQ was detected in commercially available RFP capsules, when developed without prior washing with ascorbic acid. Moreover, even though they were purified RFP standards, if they were dissolved in methanol and kept at room temperature for more than 6 h before analysis, the peak of RFPQ due to the oxidation of RFP was clearly detected.

One of the problems still remaining is the mutual separation of water-soluble conjugates of DDS and MADDS such as 4-N-glucosiduronates and 4-N-sulfamates which are respectively called DDSG [24], DDSS [25], MADDSG and MADDSS [26]. No single method of determination for these compounds can be found except for their approximate determination by stepwise hydrolysis with HCl [27]. Although these compounds are mutually separable on silica plates by developing with isopropanol—chloroform—methanol—10 *N* ammonia (10:10:5:2) [28] or with isopropanol—*n*-butanol—phosphate buffer (pH 7.4) (3:2:1) [29], further work is necessary to establish a means of quantitating them.

On the other hand, no references can be found in the literature which demonstrate any metabolite of clofazimine except a presumption based on in vitro experiments where the production of a labile leuco-type of clofazimine was detected [9, 30].

So far as we have examined, no disturbance of either the mutual separation or sensitivity in analyzing sulfone compounds due to lowering of the theoretical plate number or instability of the baseline was observed even after analyzing several tens of redissolutes containing RFPs, clofazimine and PEPL, all of which accumulated inside the column when developed by system A. Moreover, all of the compounds were eliminated during conditioning of the column by system B. The order of development from system A to B was exchangeable with that from system B to A.

Since a uniform layer could be preserved up to an increased ratio of serum to

chloroform and DMF (no higher than 3:1:5), the minimum measurable concentrations of every drug may be lowered further. At the same time, minimization of the volumes of both blood and the SEx system will be possible by the use of a micro-analytical technique; for example, the rapid separation of SExS supernatant on a micro-centrifuge following the redissolution inside a smaller test tube will achieve this purpose and will further facilitate the analysis of a number of specimens, if all of the redissolutes from the specimens contain these drugs and their main metabolites in final concentrations not less than the respective practical minimum measurable quantities.

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