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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF PYRIMETHAMINE AND RELATED DIAMINOPYRIMIDINES IN BODY FLUIDS AND TISSUES

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SUMMARY

Several 2,4-diaminopyrimidines which inhibit the enzyme dihydrofolate reductase are quantitated following extraction and separation on silica gel thin-layer chromatographic plates. These compounds are candidates for the treatment of brain tumors and meningeal leukemia, because they have the ability to cross the blood–brain barrier. The ultraviolet absorption of the pyrimidine ring at 275 nm is utilized to quantitate these compounds on thin-layer chromatographic plates with a scanning instrument. This method offers the advantages of speed, specificity, versatility and sensitivity, and has proven to be satisfactory for the measurement of as little as 10 ng/ml of these compounds in biological fluids.

INTRODUCTION

A recent communication[1] reported a sensitive analytical procedure for pyrimethamine and related 2,4-diaminopyrimidines, based upon the induced fluorescence of these compounds when sprayed with 2 *M* aqueous ammonium hydrogen sulfate. The resulting fluorescence was then quantitated with a Schoeffel TLC scanner. An improved procedure described herein, utilizing absorbance, has the advantages of speed, sensitivity and versatility over the induced fluorescence technique. It can be applied satisfactorily to substituted pyrimidines that differ considerably in the intensity of their fluorescence. The methods previously applied to the measurement of pyrimethamine in plasma and urine[2–4], are less satisfactory to support clinical pharmacokinetic studies[5].

The antimalarial drug pyrimethamine (Daraprim®)[6,7] is a folic acid antagonist that acts by inhibiting the enzyme dihydrofolate reductase[8] (EC 1.5.1.4). Following the use of pyrimethamine to treat the meningeal complications of leukemia[9], we ex-

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amined several related compounds known to be more potent inhibitors of dihydrofolate reductase[10] for their ability to cross the blood–brain barrier[5]. The development of this new improved method of analysis for 2,4-diaminopyrimidines has proven to be useful for pharmacokinetic studies in animals and for monitoring plasma and cerebrospinal fluid levels of patients during treatment.

EXPERIMENTAL

Apparatus

Fluorescence measurements were made using a Schoeffel SD-3000 spectrodensitometer at an excitation wavelength of 295 nm. The total emission above 400 nm, which had been passed through a Corning 3-74 cut-off filter, was recorded. Absorbance determinations were made with the same instrument by scanning at 275 nm without using any filtering or emission-selecting devices. The areas under the peaks of sample and reference compounds were recorded on a Honeywell Electronik 124 recorder and simultaneously integrated with an Autolab System IV integrator (Autolab, Mt. View, Calif., U.S.A.). The integrated peak areas are recorded in microvolts (counts).

Materials

All organic solvents and chemicals were analytical-reagent grade (Mallinckrodt, St. Louis, Mo., U.S.A.). The thin-layer plates used were manufactured by E. M. Reagents for Brinkman. Silica gel, F-254 plates (0.25 nm, 20 × 20 cm) were divided (scored) into twenty channels, 1 cm each, prior to sample application. Analtech (MH) plates are nearly equivalent. Plates lacking the fluorophore give equivalent sensitivity, but the fluorescent background enables easy visualization of the spots under ultraviolet light. All the work herein utilized the F-254 plates. Brinkman rectangular chromatography tanks (23 × 29 cm), backed with Whatman No. 1 filter paper, were used for development of the plates.

Procedure

Extraction from plasma and urine. The 2,4-diaminopyrimidines were extracted as follows. Plasma (1.5 ml) or urine (2 ml) containing the drug of interest, was made basic (pH > 12) by the addition of 0.1 ml of 8 *N* sodium hydroxide. 1,2-Dichloroethane (6.0 ml) was added to the sample in a 15-ml glass centrifuge tube, and the mixture was shaken for 15 min. A 5-ml aliquot of the organic phase was evaporated to dryness under nitrogen.

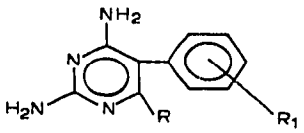
Extraction from tissues. Tissue samples (*ca.* 0.5 g) were homogenized with 5 ml of 2 *N* lactic acid using a Duall tissue grinder. After centrifugation at 35,600 *g* for 20 min, 2.0 ml of the supernatant were transferred to 45-ml centrifuge tubes followed by the addition of 1.0 ml of 8 *N* sodium hydroxide and 15 ml of 1,2-dichloroethane. The samples were shaken for 15 min, centrifuged at 1500 *g* for 10 min, and then a suitable aliquot of the organic phase was evaporated to dryness under nitrogen.

Thin-layer chromatography. The residue from evaporation was redissolved in 0.1 ml of chloroform and a suitable aliquot (20–80 μl) was spotted on a prescored, silica gel, F-254 plate. Spot application was achieved using an electronically driven "TLC multi-spotter" (Analytical Instrument Specialties, Anaheim, Calif., U.S.A.).

Standards of known amounts were also spotted on the same plate. After application of the sample and the standards, the plate was developed in a chloroform–methanol (70:30) system, to a height of 15 cm. The R_F values for the various 2,4-diaminopyrimidines ranged between 0.5 and 0.7 (Table I). The solvent system was prepared fresh each morning and allowed to equilibrate for at least 3 h. The plate was developed for 1 h and was then allowed to air dry for several minutes, after which time it was scanned and each zone quantitated.

TABLE I

STRUCTURES AND R_F VALUES OF 2,4-DIAMINOPYRIMIDINES INVESTIGATED



Compound	*R*	R_1	*Name*	R_F
I	C_2H_5	4-Cl	pyrimethamine, Daraprim®	0.49
II	CH_3	3,4-Cl_2	DDMP	0.52
III	C_2H_5	3,4-Cl_2	DDEP	0.58
IV	C_2H_5	3-NO_2, 4-Cl	—	0.61

RESULTS AND DISCUSSION

Initial studies with several of the 2,4-diaminopyrimidines indicated that their fluorescence on the TLC plates can be amplified several-fold by spraying with 2 *M* ammonium hydrogen sulfate[1]. Because considerable variation in the intensity of fluorescence was observed from one analog to another in this series, it became evident that a more versatile approach was needed for the assay of those compounds that did not respond to the ammonium hydrogen sulfate spray or that were weakly fluorescent. All compounds shown in Table I were readily detected by their ultraviolet absorbance at 275 nm on TLC plates impregnated with a fluorescent phosphor. This method is more sensitive and less variable than the fluorescent technique, and is applicable to all compounds in the series regardless of their ability to fluoresce.

A comparison of the fluorescence and absorption procedures for compounds I–IV is shown in Fig. 1. Because the energy output of the excitation light varies with wavelength, the power output to the photomultipliers was normalized so that a direct comparison of the two procedures could be made. The dotted line represents the mean regression slope for all four compounds, as determined by their ultraviolet absorbance at 275 nm. The range at each point is also shown. The same plate was then sprayed with a 2 *M* ammonium hydrogen sulfate solution as described[1]. Because the fluorophore in the F-254 plates is completely destroyed by this reagent, the fluorescence of the same compounds was then determined at 295 nm. The difference in the slope of the plot between pyrimethamine (compound I) and the nitro analogue (compound IV)*

* This compound was prepared in these laboratories by Elvira A. Falco, by the nitration of the corresponding chlorophenyl pyrimidine. The structure of IV was proven by oxidation to the known benzoic acid derivative.

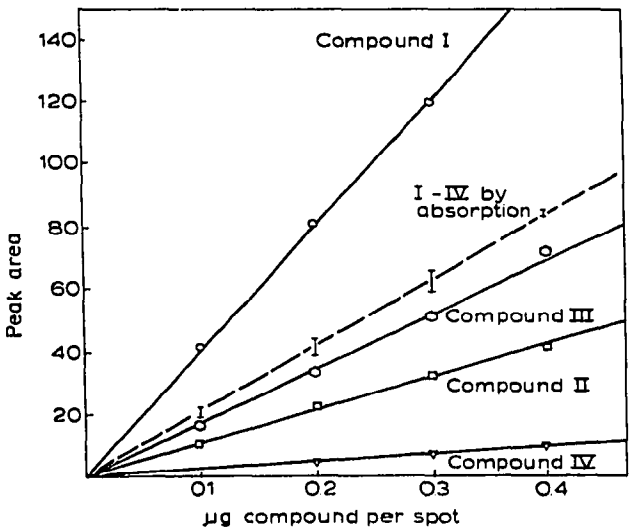


Fig. 1. Variation in sensitivity for compounds I–IV, when measured by the fluorescence (———) and absorption (— — —) modes.

illustrates the marked variation in the sensitivity of the different compounds when measured by the fluorescence method. Other 2,4-diaminopyrimidines from a larger series of analogs investigated also produced marked differences in their fluorescence, but were uniformly consistent in their ultraviolet absorption characteristics. The nearly identical slopes of compounds I-IV, when measured by absorbance, suggested that the 2,4-diamino moiety was responsible for their ultraviolet absorption. Indeed, further studies with other substituted and non-substituted pyrimidines at 275 nm indicated that the moiety responsible for absorbance was the pyrimidine ring. Spectral data (Table II) list a $\lambda_{max.}$ of 266 nm for 2,4-diaminopyrimidine in the lower pH ranges. Because silica gel plates are themselves slightly acidic (pH *ca.* 4.0), it is likely that the protonated species is being observed at 275 nm.

TABLE II

ULTRAVIOLET SPECTRAL DATA FOR PYRIMIDINES AND AMINOPYRIMIDINES

Compound	*Solvent or pH*	$\lambda_{max.}$
Pyrimidine[12]	pH 7	243, 271
	CH_3OH	239, 243, 279
	4 *N* H_2SO_4	242
Pyrimidine[13], 2-amino	H_2O	224, 292
4-amino	H_2O	236, 273
Pyrimidine[13,14], 2,4-diamino	pH 10.4	228, 282
	pH 1.0	266
	HCl	232*, 266
	H_2O	227, 282

* s = Shoulder.

It should be noted that the presence or absence of a fluorescent indicator in the plates neither enhances nor diminishes the recorded peak areas of the various analogs. Thus, for these compounds, both types of plates are satisfactory for absorbance measurements. Although fluorescence layers were used, it is important to stress that ultraviolet absorbance, and not fluorescence quenching was being measured.

Fig. 2 compares the scans of compound II (DDMP) in the absorbance and fluorescence modes. Note particularly, the increase in the noise level of the fluorescence mode. The low quantum efficiency of the fluorescent species combined with a decrease in photomultiplier sensitivity at longer wavelengths, required a substantial increase in photomultiplier sensitivity, resulting in a marked increase in background noise.

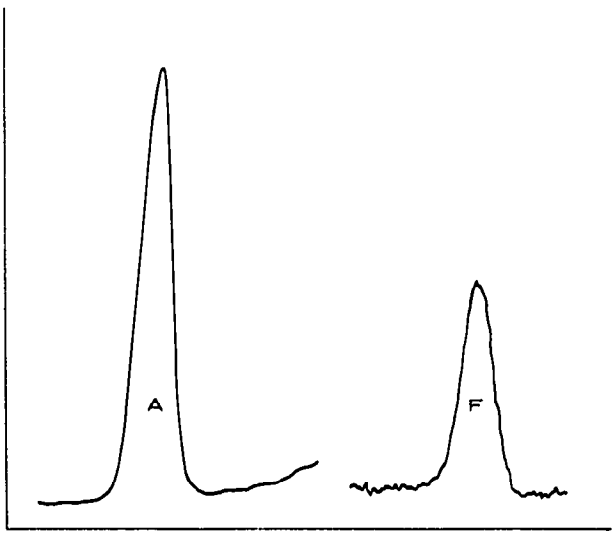


Fig. 2. Tracing of strip chart scan of compound II (DDMP) in the absorption (A) and fluorescence (F) modes.

The sensitivity of the absorption method is illustrated in Fig. 3. As an example, a linear regression of peak area *vs.* mass is shown for compound II from 10 to 250 ng per spot. This sensitivity allows quantitative analysis of 5–10 ng of drug per milliliter of sample. An additional advantage of the absorption method is its linear dynamic range. Deviations from linearity occur at approximately 400 ng per spot with the fluorescence spraying technique, but the linear range is extended from 10 to 800 ng per spot with the absorption method.

To check the precision of the procedure, six separate aliquots of a urine collection from a patient receiving compound II were assayed. All six individual samples were carried through from extraction to quantitation. Table III lists the integrated counts (in microvolts), as determined from single scans of each sample. The precision proved to be satisfactory with a standard deviation of only 2.27 %.

Previous experiments performed with pyrimethamine and DDMP have demonstrated that these drugs can be completely recovered from body tissues and

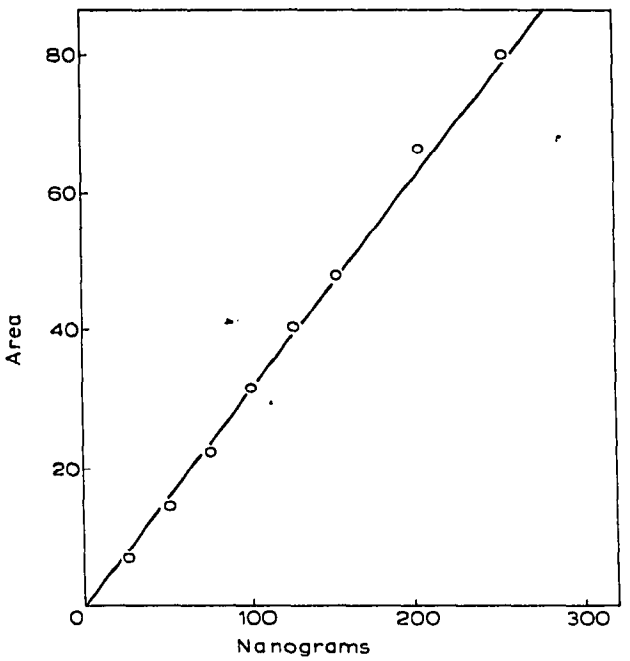


Fig. 3. Calibration curve for DDMP.

fluids. While earlier recovery trials relied on the induced fluorescence method, the more recent use of absorbance scanning would not be expected to alter this recovery. To confirm that the total assay procedure would not be compromised if ultraviolet absorbance were employed, a direct comparison was made. Various amounts of these drugs were added to plasma and carried through the analytical procedure. After initial quantitation of the compounds by the absorbance method, two of the plates were sprayed with the hydrogen sulfate reagent, allowed to air dry, and quantitated by the fluorescence mode. As shown in Table IV, identical results were obtained by the two modes of analysis.

TABLE III

PRECISION OF THE ANALYTICAL PROCEDURE FOR THE DETERMINATION OF COMPOUND II IN URINE

Sample No.	*Peak area*
1	175.4
2	187.0
3	183.6
4	182.3
5	180.6
6	178.0
Mean	181.15
S.D.	4.12 or 2.27%

TABLE IV

COMPARISON OF THE ABSORBANCE AND FLUORESCENCE MODES FOR THE DETERMINATION OF THE RECOVERY OF COMPOUNDS I–III FROM PLASMA

Drug added (μg/ml)	*Recovered (μg/ml)*	
	Absorption	*Fluorescence*
Compound I		
0.40	0.39	0.43
0.60	0.58	0.59
0.87	0.90	0.88
1.00	1.03	1.10
1.20	1.17	1.17
Av. (%)	99.7%	103.0%
Compound II		
0.16	0.17	—
0.20	0.19	—
0.28	0.29	—
0.32	0.30	—
0.40	0.37	—
0.48	0.49	—
Av. (%)	100%	—
Compound III		
0.20	0.19	0.19
0.40	0.43	0.40
0.60	0.59	0.58
0.80	0.77	0.82
1.00	0.93	1.06
1.20	1.17	1.25
Av. (%)	97.9%	100.5%

A similar absorption scanning method for assaying trimethoprim [2,4-diamino-5-(3′4′5′-trimethoxybenzyl)pyrimidine] and sulfamethoxazole in plasma has been described by Sigel *et al.*[11] of this laboratory.

The absorbance procedure offers several advantages over the induced fluorescence spraying technique. The TLC plate can be quantitated within minutes after removal from its development tank. The necessary caution required in spraying a hazardous chemical evenly on the surface of a TLC plate is avoided and the extra hour needed for drying is eliminated. It should be emphasized that the upper linear range for quantification on the plates can be increased from 0.4 to 0.8 μg per spot by using the absorption method. This is particularly important with urine samples, because their drug concentrations can vary greatly. From our experience with the 2,4-diaminopyrimidines, it is clear that many more of these compounds can be determined with greater sensitivity by absorbance than by induced fluorescence.

These advantages are exemplified by the work that has been done monitoring the drug-plasma concentrations of pyrimethamine in patients during the course of treatment. The application and usefulness of this procedure is illustrated in Fig. 4,

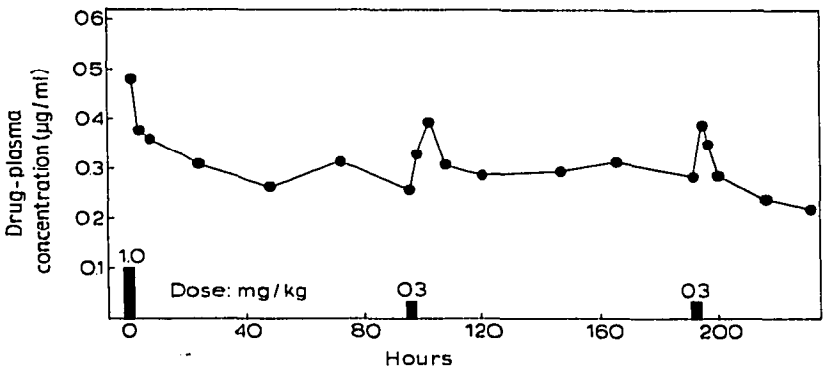


Fig. 4. An example of monitoring plasma levels of a patient treated with pyrimethamine.

which is part of a study[5] relating drug-plasma concentration to dosage and frequency of administration. The TLC scanning procedure is an excellent method of monitoring drug-plasma levels to define kinetic characteristics and to avoid potentially toxic effects.

CONCLUSION

A specific quantitative TLC procedure has been described for several 2,4-diaminopyrimidines, and a general procedure for quantitating a wide variety of compounds incorporating the pyrimidine moiety. The selectivity of TLC is combined with the sensitivity of ultraviolet absorbance scanning. This combination has proven most suitable for the determination of drugs in body fluids and tissues and for defining pharmacokinetic parameters.

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