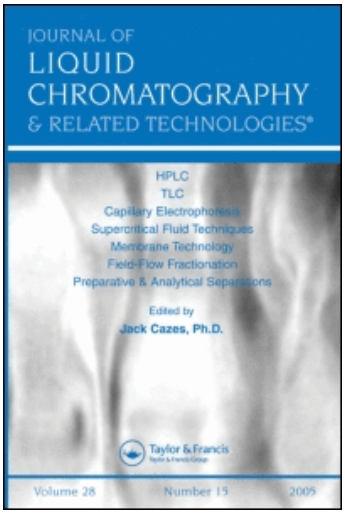

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Thin Layer Chromatographic Method for Rapid Quantification and Identification of Trimethoprim and Sulfamethoxazole in Pharmaceutical Dosage Forms

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THIN LAYER CHROMATOGRAPHIC METHOD FOR RAPID QUANTIFICATION AND IDENTIFICATION OF TRIMETHOPRIM AND SULFAMETHOXAZOLE IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

A densitometric and a spectrophotometric method for rapid but accurate determination of sulfamethoxazole (SMA) and trimethoprim (TMP) present in combined dosage forms were described. SMA and TMP were extracted with 90% acqueous methanol and the interfering and related contaminants were removed by thin layer chromatography (TLC) or high performance TLC (HPTLC) on silicagel plates using chloroform : isopropanol : diethylamine :: 10 : 6 : 1 (v/v) as mobile phase. Assay was done at the respective absorption maxima of the drugs by in situ densitometry and by spectroscopy after extracting the drugs from TLC plates with 90% acqueous ethanol. Results obtained by both the methods agreed well with those obtained by the method prescribed by the United States Pharmacopoeia XXI edition. Total time required for HPTLC and densitometric assay of 32 samples using 4 standards was 30 min. Probable source of errors in densitometric studies and their rectification was discussed.

INTRODUCTION

Combination of Trimethoprim (TMP) and Sulfamethoxazole (SMA) is marketed as tablets (conventional, dispersible or paediatric), injections or liquid oral preparations. Determination of TMP and SMA in these dosage forms have been described by different compendia (1,2,3). The asay procedures involve either separation of the active components by high performance liquid chromato-

graphy (HPLC) followed by measurement of absorbance at 254 nm for both the components (3) or, determination of the components individually (1,2) by spectroscopy after chloroform extraction (TMP), and amperometric titration (SMA). These methods are good in accuracy but are manipulatively difficult, time consuming and not suitable for analysis of a large number of samples at a time. Spectrophotometric (4) and colorimetric (5) methods for rapid estimation of these compounds have been described but both the methods are non-specific with respect to related compounds and break down products, and the former is prone to interference from stabilizers. TMP and SMA may easily be separated from one another and from their related compounds, break down products, preservatives etc by thin layer chromatography (TLC) (1,2,3). Since a large number of samples may easily be chromatographed simultaneously on a single plate using a small amount of solvent, TLC is a highly economical as well as rapid method for determination and identification of active principles in drug substances. Introduction of high performance TLC (HPTLC) and modern zigzag scanning densitometers has made the assay procedure more rapid, highly reproducible, simple and cost effective. The present paper describes a spectrophotometric and a densitometric method for quantitation of TMP and SMA after separation from probable interfering substances by TLC or HPTLC.

MATERIALS

Apparatus

a) Densitometer - Shimadzu dual wavelength TLC scanner, Model CS-930.

b) Spectrophotometer - Shimadzu UV-VIS double beam recording spectrophotometer Model Graphicord UV-240.

c) HPLC System - Waters HPLC system equipped with U6K universal injecter, solvent delivery system Model 45, Model 440 UV/VIS detecter and omniscribe single pen recorder. Chromatography was carried out on a μ Bondapack C18 column.

d) Ultraviolet Viewer - Desaga UVIS system.

e) Sample applicator - Camag Nanomat II with nanopipette 100 nl and micropipette 1 μl.

f) Table Centrifuge - (Swing head, 5000 rpm) Model - Remi R8C.

g) TLC plates - 20 X 20 cm, coated with 0.4 mm thick layer of silicagel E. Merck; Silicagel 60G : Silicagel HF 254 :: 10 : 3 (w/w) Activated at 110^0C for 1 hr.

h) HPTLC plates - 10 X 10 cm Kieselgel 60 F254 (E.Merck), activated at 110^0C for 30 min.

i) TLC chamber - Camag Twin trough tanks for 20 X 20 cm and 10 X 10 cm plates.

Reagents

Chemicals : Sulfamethoxazole and Trimethoprim were USP reference standards., sulfanilamide, sulfanilic acid and sulfamethoxazole N 4 - glucoside were Central Drugs Laboratory reference standards. Chloroform, methanol and isopropanol were of GR, E.Merck grade, diethylamine was distilled over alkali and dehydrated ethanol was of USP grade.

Mobile phase

a) TLC - Chloroform : Isopropanol : Diethylamine :: 10 : 6 : 1 (v/v).

b) HPLC - As per USP (3).

Standard solutions - The following reference standards were dissolved in 10 ml of methanol : water :: 9 : 1 and used for TLC and HPTLC.

a) SMA (25 mg) and TMP (5mg)

b) SMA (50 mg) and TMP (10 mg)

c) SMA (100 mg) and TMP (20 mg)

d) Sulfanilamide (5 mg)

e) Sulfanilic acid (5 mg)

f) Sulfamethoxazole - N4-glucoside (5 mg)

g) SMA (50 mg), TMP (10 mg), sulfanilamide (5 mg), sulfanilic acid (5 mg) and sulfamethoxazole- N4-glucoside (5 mg).

PROCEDURE

Twenty tablets were weighed and ground to a fine powder. Powder equivalent to 50 mg of SMA and 10 mg of TMP was transferred into a 10 ml volumetric flask and 1 ml of water was added followed by shaking on a vortex mixer for 1 minute. Methanol (5 ml) was then added to it and warmed briefly (30 seconds) on a steam bath with continuous shaking. The mixture was then cooled to room temperature, volume was made up with methanol, mixed throughly and the residue was allowed to settle (or it may be centrifuged if required). The clear supernatant was ready for TLC or HPTLC.

For assay by scanning densitometry, 1 µl of the test solution and 1 µl of the standard solution (b) were applied as separate compact spots 10 mm apart on an imaginary line 15 mm from the bottom of a TLC plate. For HPTLC, 100 nl of the sample or standard was applied on a line 10 mm from the bottom, the lane distance being 5 mm. The plate was developed up to 50% of total length of the plate in the usual way in a filter paper lined tank previously saturated with the developing solvent for 1 h and containing 15 ml or 5 ml of the mobile phase in each trough for TLC or HPTLC respectively. After chromatography was complete, the plates were dried in a current of warm air for 10 min. SMA and TMP spots were visualised under short wave UV and the starting and end

points for densitometry of the first lane were marked. The plate was then scanned in the densitometer with liniarizer setting at X=3, back ground scanning wavelength at 320 nm and sample scanning wavelength at 257 nm (SMA) or 288 nm (TMP and SMA). Other parameters were set as per the instruction mannual of the instrument.

To study the linearity of area value with concentrations, standard solution a,b and c were estimated by the above densitometric method.

To study the solvent suitability and resolution, standard b,d,e,f and g were chromatographed as above and the chromatogram for sample g was recorded both at 288 nm and 257 nm.

For spectrophotometric assay after TLC, 10 µl of each of the sample and the standard solution b was applied as separate 5 mm wide bands on an ordinary silicagel plate on a line 20 mm from the bottom. Plates were then developed as described before except that the mobile phase was allowed to ascend 120 mm from the starting line. SMA and TMP were then visualized under short wave UV lamp as before and their positions were marked with a needle. Each silicagel band containing the desired material were scrapped into separate 15 ml stoppered centrifuge tubes and extracted twice with ethanol (3 ml + 2 ml for SMA and 2 ml + 1 ml for TMP) by shaking on a vortex mixer for 30 seconds followed by centrifugation at 2000 g for 5 minutes. A silicagel blank obtained by extraction of silicagel from an area equivalent to respective sample bands but not containing any sample gave very low absorption value at the wave lengths used. The silicagel blank may be used to replace the alcohol blank if desired but the solvents and silicagel should be checked for purity if the silicagel blank shows a high absorbance (above 0.010).

HPLC was carried out as per USP XXI (3).

RESULTS

The proposed methods were validated by comparing the results of the TLC-spectrophotometry and TLC-densitometry analysis with those obtained by an official method (USP XXI). Table 1 and 2 show the assay results of sulfamethoxazole and trimethoprim respectively from 10 different TMP-SMA tablets using proposed and official methods. There is a good agreement between the results obtained by all the methods. Low standard deviation (USP XXI allows 2%) indicates high precision and reproducibility of the proposed methods. The assay values obtained for five SMA-TMP tablets by lane wise scanning (in the direction of solvent migration) and transverse scanning (perpendicular to the direction of solvent migration) on an HPTLC plate did not differ from one another significantly (Table 3). Table 4 indicates the relation of quantity of sample applied

TABLE I.

Assay values of Sulfamethoxazole (Percent label claim) in Commercial Sulfamethoxazole - Trimethoprim Tablets obtained by the Proposed and Official Methods.

	Proposed methods								HPLC[b]
	Densitometry after[a]						Spectroscopy after TLC[a]		USP
	TLC		HPTLC						
			285 nm		257 nm				
	Average	SD	Average	SD	Average	SD	Average	SD	Average
1.	105.70	0.72	104.85	0.88	104.62	0.80	102.30	1.56	103.9
2.	103.00	1.87	102.88	1.48	102.45	1.09	101.91	1.07	102.6
3.	101.18	2.40	100.90	0.20	101.06	0.81	101.71	1.30	100.2
4.	100.42	2.08	98.80	1.90	99.30	1.32	99.44	1.16	98.9
5.	97.65	1.19	98.38	1.10	98.32	1.14	99.40	1.10	98.8
6.	97.08	1.39	98.13	2.12	98.24	2.01	97.58	0.41	98.8
7.	96.77	1.45	96.96	0.25	97.06	0.58	96.09	0.22	97.6
8.	95.08	0.79	96.38	0.75	96.69	1.02	96.41	0.79	97.6
9.	94.42	1.28	94.14	0.12	93.76	1.03	92.36	1.41	93.5
10.	90.16	1.30	89.93	0.86	89.51	0.66	89.01	0.92	90.5

a. Average of 6 independent determinations
b. USP limit for SD is 2%.
c. SD - Standard Deviation.

TABLE 2.

Assay Values of Trimethoprim (Percent label claim) in Commercial Sulfamethoxazole - Trimethoprim Tablets obtained by the Proposed and Official Method.

	Proposed method						
	Densitometry after[a]						
	TLC		HPLC		Spectroscopy after TLC[a]		HPLC[b] (USP)
	Average	SD	Average	SD	Average	SD	
1.	102.08	1.36	103.81	0.82	102.3	1.56	103.3
2.	101.31	1.32	100.76	0.44	101.9	2.07	101.4
3.	101.30	2.02	100.42	0.94	101.7	2.3	100.2
4.	100.34	1.65	99.24	1.18	99.4	2.1	98.4
5.	99.36	0.63	99.24	1.96	99.4	1.1	98.4
6.	98.88	0.44	98.89	0.85	97.58	1.04	98.4
7.	98.31	0.58	98.36	0.99	96.09	1.22	96.5
8.	97.98	0.64	98.16	1.84	96.41	0.79	97.7
9.	94.07	2.0	92.04	1.97	92.36	1.41	92.8
10.	92.87	0.13	89.75	1.35	89.01	1.92	90.8

a. Average of 6 independent determinations.
b. USP limit for SD is 2%.

TABLE 3

Assay Values (Percent label claim) obtained by Lane Wise and Transverse Scanning Densitometry of Some Sulfamethoxazole - Trimethoprim Tablets*.

	Sulfamethoxazole (at 257 nm)				Trimethoprim (at 288 nm)			
	Transverse scanning		Lane Wise scanning		Transverse scanning		Lane Wise scanning	
	Average	SD	Average	SD	Average	SD	Average	SD
1.	104.39	0.30	104.62	0.80	104.19	0.92	103.81	0.82
2.	102.13	1.34	102.45	1.09	101.06	0.63	100.76	0.44
3.	99.02	0.43	99.30	1.32	98.39	1.00	99.24	1.18
4.	96.95	1.46	97.06	0.58	96.60	0.84	98.36	0.99
5.	90.65	0.36	89.51	0.66	90.16	1.44	89.75	1.35

*Average of 6 independent determinations.

TABLE 4.

Linearity of Concentration of Sulfamethoxazole and Trimethoprim with Area Values obtained by Densitometry after TLC*.

Sulfamethoxazole				Trimethoprim			
Amount applied (μg)	Integrated area value		r	Amount applied (μg)	Integrated area value		r
	Average	SD			Average	SD	
2.5	57112	1371		0.5	28720	574	
5.0	112853	1599	0.9993	1.0	57440	1723	0.9997
10.0	228220	6853	($p<0.02$)	2.0	114880	1723	($P \ll 0.01$)

*Average of 6 independent determinations.

with integrated area value on an ordinary TLC plate. The r value indicates highly significant linear relation under the experimental conditions. The reflection absorption spectrum of SMA and TMP (Fig.1) is same as the absorption spectrum of ethanolic solution of these two compounds. Absorption maxima of SMA and TMP are 257 and 288nm respectively and neither of them has any absorption at 320 nm. Thus for hand drawn plate, scanning the sample containing zone at 320 nm served as control for elimination of error due to possible unevenness of the plate. For maximum sensitivity, TMP and SMA were assayed at their respective absorption maxima in spectrophotometric or densitometric studies. However, since SMA concentration is usually five times that of TMP, assay of the former one at 288 nm did not show any loss of accuracy (Table 1). Fig. 2 shows a typical densitogram of a sample mixture containing sulfamethoxazole-N4-glucoside (100 ng), sulfanilic acid (100 ng) sulfanilamide (100 ng), SMA (5 µg) and TMP (1 µg). The contaminants are clearly separated from SMA and TMP in the chromatographic system used and did not interfere with the assay even at 10 times the permissible limit set by USP XXI (3).

DISCUSSION

Quantitative analysis of drug substances present in a combined dosage form needs attention to two important factors viz. (a) separation of the active ingradients from one another and from probable interfering related and breakdown products and (b) elimination of errors due to poor recovery during the separation. Pharmacopoeial procedures for assay of SMA and TMP generally include adequate precautions in respect of both 'a' and 'b' (1,2,3). However, during analysis of a large number of samples by these methods, errors may be introduced inadvertently due to multiplicity of steps and complicacy of the procedures. An ideal process for rapid but accurate quantitative analysis should allow processing of a large number of samples at a time for separation of the target materials from interfering substances and assay the materials using a simple, preferably single step procedure. Quantitative analysis by scanning densitometry after TLC is an ideal process in these respects but attention must also be given to the facts that, unlike spectroscopy of solutions, reflection absorbance of a sample is not linearly proportional to the concentrations of the sample. This happens because of non uniform density of the sample spot across the measuring beam (6) and non-linear relationship between relfactance and sample concentration as predicted by Kubelka-Munk theory (6). These problems may be avoided by measuring only the central part of the sample spot which should preferably be a small band, produced by applying the sample by sprarying as an uniform band,

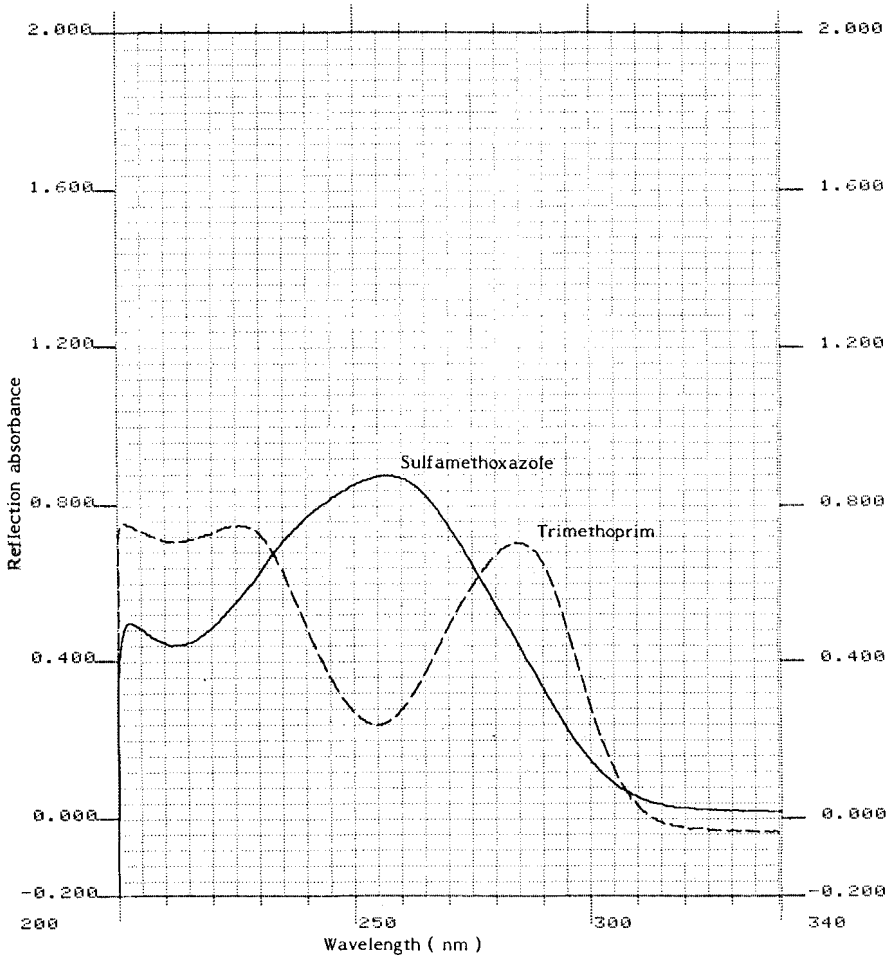

Fig 1. Reflection absorption spectra of Sulfamethoxazole and Trimethoprim.

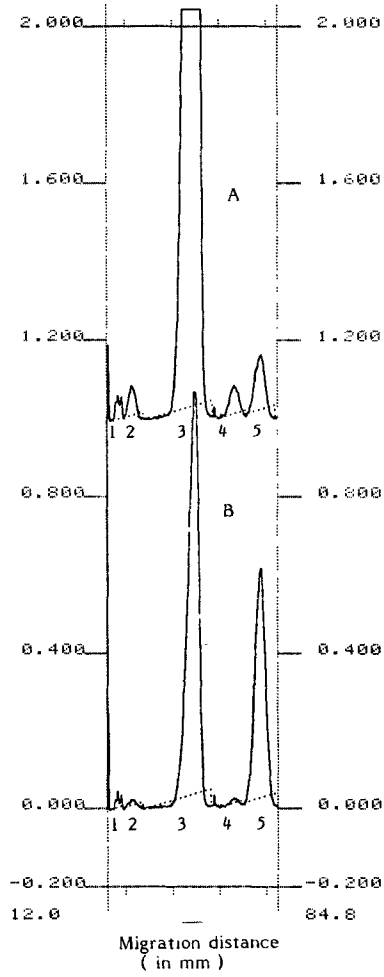

Fig. 2 Separation of Sulfamethoxazole and Trimethoprim from related products by TLC. Scanning was done lane wise at 257 nm(A) and 288(B).

1) Sulfamethoxazole - N4 glucoside (100 ng)

2) Sulfanilic acid (100 ng)

3) Sulfamethoxazole (10 µg)

4) Fulfanilamide (100 ng)

5) Trimethoprim (1 µg)

and by working within a narrow concentration range. However, this will necessitate scanning of all the samples individually as scanning perpendicular to the direction of development (transverse scanning) is not possible here and at least three different concentrations of standards (50%, 100% and 200% of the expected concentration of the sample) will be needed to attain reasonably good result. A more satisfactory way of solving these problems is to scan the whole spot with a very small beam of light and linearize the relationship between reflection absorption and concentrations by using a working curve linearizer programmed by a microcomputer on the basis of Kubelka-Munk theory. We have used a densitometer of this type and hence could successfully eliminate density variations across the scanning beam and variation in concentrations did not affect the computed value. Also since the whole spot was scanned, same area values are obtained by transverse or longitudinal scanning. In spectrophotometry after TLC, concentration is linearly related to absorbance over a wide range and sample solutions of widely different concentrations may accurately be estimated using a limited number of reference standards or a working standard curve. However, extraction of the sample or standard from adsorbent (silicagel) is needed to be quantitative here.

In our method, the sample preparation step is simple, use of TLC allows separation of all the probable interfering compounds (as listed by USP XXI) even when present at a concentration 10 times the USP limit. An extremely large number of sample may be chromatographed on the plates used by us by applying samples on two opposite sides and developing the plate from both ends simultaneously (HPTLC) or in steps (HPTLC or TLC). We could analyse 36 samples on a single plate and assay them by densitometry. In spectrophotometric determination the number of usable tracks reduces to 18 since larger volume of a sample was to be applied here but even then the number was large enough to meet the need of most laboratories. The TLC - spectrophotometry is a highly accurate method as indicated by extremely low SD values and in our laboratory, an analyst could handle about 50 samples a day using this method. The TLC-Densitometry method though showed marginally higher percentage of standard deviation, it was still much lower than the allowable pharmacopoeial limit for SMA-TMP combinations (3). The total time required to estimate all the samples (36 SMA and 36 TMP spots) using single beam and linear scanning mode was only 5 minutes for HPTLC and in single beam zigzag scanning mode, it was 20 minutes. The time taken for sample application (10 min.) and development (15 min.) of such a plate was also very short. Since solvent requirement for HPTLC is only 5 ml, the densitometric assay described by us was an extremely rapid and economical method for quantitation of SMA and TMP without sacrificing accuracy.

However, the type of densitometer suitable for these analysis is somewhat costly and in laboratories where it is not available, the TLC-spectrophotometric method, which was also fast, economical and very accurate, may be used.

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