SIMULTANEOUS DETERMINATION OF SULPHAMETHOXAZOLE AND TRIMETHOPRIM IN CO-TRIMOXAZOLE TABLETS USING RP-LC ANALYSIS

N. N. A. Okine, S. Asare-Nkansah and A. Acheampong
Department of Pharmaceutical Chemistry,
Faculty of Pharmacy, Pharmaceutical Sciences, College of Health Sciences
Kwame Nkrumah University of Science and Technology,
Kumasi, Ghana

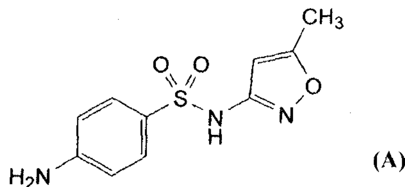
ABSTRACT

A reversed phase liquid chromatographic (rp-lc) method has been developed to determine simultaneously the amount of sulphamethoxazole and trimethoprim in co-trimoxazole tablets using salicylic acid as internal standard. The chromatographic resolution was achieved with 96% ethanol and 0.1M ammonium perchlorate buffer (95:5) on a reversed phase column at ambient temperature. UV detection was at 254 nm. The flow through the column was 0.8ml/min with a chart recorder speed of 5mm/min. Mean retention times for trimethoprim sulphamethoxazole, and salicylic acid were 3.4 ± 0.13 min, 3.8 ± 0.08 min, and 4.5 ± 0.13 min respectively. Calibration curves were linear over the ranges 0.0001-0.0006%$^{w}/_{v}$ (sulphamethoxazole) and 0.002-0.012%$^{w}/_{v}$ (Trimethoprim). The method has been used to analyze a branded co-trimoxazole (Septrin) tablets and a locally formulated co-trimoxazole tablets. The percentage content of sulphamethoxazole and trimethoprim determined by the rp-lc and the official u.v methods were found to be comparable and within the BP 2000 standards. Comparing this work with that of Ronn et al 1999, revealed that, this method is cost effective for routine analytical work as the mobile phase is devoid of acetonitrile, an expensive reagent. Additionally, running time for the current method (4 min) is shorter than that of Ronn et al (7 min), for in-process and finished product quality monitoring.

Keywords: *Septrin, Co-trimoxazole, Sulphamethoxazole, Trimethoprim, Salicylic Acid, Rp-lc and Internal Standard*

INTRODUCTION

Rp-lc, an aspect of high performance liquid chromatography (hplc) is a separation technique that uses a column packed with a stationary phase through which a mixture of solutes is injected. Separation is effected when a liquid mobile phase is passed through the column under pressure. The separation technique is based on adsorption, partitioning, ion exchange and gel-permeation. In the case of rp-lc the stationary phase is made of polar material.



(A)

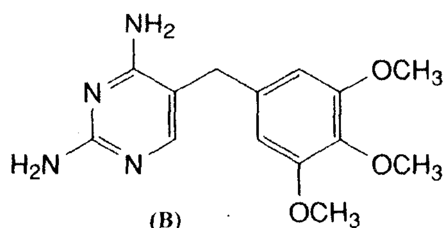


Fig. 1: Chemical structures of Sulphamethoxazole (A) and Trimethoprim (B)

Co-trimoxazole is a combination drug made up of sulphamethoxazole and trimethoprim and according to the Committee on Safety of Medicines (CSM), it is the drug of choice for the treatment of *Pneumocystis carinii* pneumonia and also considered for use in acute exacerbation of chronic bronchitis and infections of the urinary tract (BNF, 2003). Due to the Ministry of Health (M.O.H.)/WHO policy on the use of generics to keep the cost of drugs low, (GNDP, 1999 and WHO, 1999) several of these products (locally & imported especially those of Asian origin) have flooded the Ghanaian market. It has therefore become necessary to screen such products for their quality.

Analysis of several drugs via hplc methods including rp-lc have been reported in various journals (Altum *et al.*, 2001 and Erk *et al.*, 2001). It is suggested that the method is quick, reliable and above all very accurate.

The official UV spectroscopic method of analysis for co-trimoxazole involves extraction of the active components using acetone for sulphamethoxazole and chloroform for trimethoprim (BP, 2000) which is laborious. It is envisaged that development of rp-lc method which is cheap, fast and reliable may strengthen the control authorities in monitoring the several generic products on the Ghanaian market.

MATERIALS AND METHODS

Instrumentation

The liquid chromatograph utilized was Spectra Systems P100 analytical pump combined with an external 20μl Rheodyne 8125-095-syringe loading sample injector, Phenomenex (Ultracarb 5 ODS (3D); (250 x 4.6mm)) HPLC column and an Applied Biosystems 783A Programmable Absorbance UV detector. Chromatogram integration was carried out with a Shimadzu CR501 Chromatopac. UV analysis was done with a double beam Cecil 7020 UV-Visible Spectrophotometer.

Reagents

Ethanol (96%v/v, BDH), ammonia solution (28% ^{V}/v, BDH), perchloric acid (70%v/v, BDH) caffeine (BDH), salicylic acid (BDH), paracetamol (Weisheng Pharmaceuticals Company Limited, China), methanol (BDH), pure sulphamethoxazole (Indukern Chemie AG, Wiesenstrasse 33 Switzerland) and trimethoprim (Indukern Chemie AG, Wiesenstrasse 33, Switzerland) powders.

EXPERIMENTAL

Preliminary tests

In order to establish the identity and purity of pure powders and tablet samples, various Pharmacopoeial assessments were carried out. These include; colour reaction tests, melting point determination, thin layer chromatography (TLC), friability and uniformity of weight tests. Also, uv-visible spectra of both sulphamethoxazole and trimethoprim extracted from the tablets were recorded to establish their identity.

Development of rp-lc Method of Analysis

Development of the separation technique

The physico-chemical properties of the components of co-trimoxazole were searched. Information on solubility, chemical structures, acid dissociation constants, level of purity and UV profiles in acidic, basic and neutral media were sought (Moffat, 1986; BP, 2002). Due to the moderately polar nature of the compounds under investigation, the reversed-phase mode of hplc was found applicable. A reversed phase column was thus selected and a suitable polar mobile phase investigated so that under the separation, a

more polar solute was eluted before the relatively nonpolar.

Various combinations of solvents for the mobile phase were tried (. Methanol (99.8%): acetic acid (1%$^{v}/_{v}$), methanol: 0.01M Na_2HPO_4 or 0.01M NaH_2PO_4) in varying proportions. Ethanol (96%) and 0.1M ammonium perchlorate (NH_4ClO_4) buffer in a ratio of 95:5 was found the most suitable to give the best elution profile in a reasonable running time such that, the retention times of trimethoprim and sulphamethoxazole were 3.4 and 3.8 minutes respectively.

Selection of an internal Standard

A number of chemicals including caffeine, paracetamol and salicylic acid were screened to get an internal standard for the study. Aqueous solutions of the target internal standards were prepared and respectively chromatographed to obtain their respective retention times using the mobile phase already described. Paracetamol, caffeine and salicylic acid gave separations, resolutions and retention times that had the potential to be considered for an internal standard. A solution of a mixture of sulphamethoxazole, trimethoprim and paracetamol in equal proportions made up to a specified volume was prepared. The process was repeated to get two other solutions of similar composition but respectively replacing paracetamol with caffeine and salicylic acid. Aliquots (20µl) of these respective solutions were successively introduced onto the column using the same chromatographic conditions that were used to run the components individually. Among the three targets, the chromatogram of salicylic acid gave the best separation, resolution and retention time in the presence of both sulphamethoxazole and trimethoprim. It was thus selected as the internal standard for the work. The concentration of a solution of salicylic acid (internal standard) that could give peak area ratio (peak area of test drug/peak area of internal standard) of approximate unity was then investigated.

Selection of UV detection wavelength

Since the mobile phase for the study was acidic (pH 3.0), it was necessary to investigate the uv-absorption pattern (UV spectrophotometer) in an acidic environment for the drug samples under review in order to determine their respective wavelengths of maximum absorption which were approximately 265, 271 and 260 nm respectively for sulphamethoxazole, trimethoprim and salicylic acid. Though the mobile phase had no significant absorption (wavy baseline with no peaks) at any of the above wavelengths when it was injected onto the column and eluted, 254 nm was used as the detecting wavelength after further work for all the chromatographic applications because at that wavelength, a relatively steadier baseline was obtained. In addition, all components of a solution had significant detection that was useful quantitatively.

Selection of Flow Rate and Chart recorder speed

Among other things, the retention times of solutes on chromatographic columns are affected by the rate of flow of mobile phase through the column. The flow was therefore adjusted till a value that gave significant solute retention on the column in the minimum amount of time for both drug samples and internal standard was obtained (0.8 ml/min.). After further investigations, a recorder speed of 5mm/min was adequate to reasonably space out chromatograms on the recorder chart.

Preparation of solutions

Solutions for calibration curve

Pure sulphamethoxazole (400mg) and trimethoprim (80mg) were weighed into a 100ml volumetric flask (to mimic content of a tablet). Sufficient ethanol (96%) was added to dissolve the two components and the mixture topped up to volume with the solvent. Aliquots of 2.5ml, 5.0ml, 7.5ml, 10ml, 12.5ml and 15ml were pipetted into six different 100ml volumetric flasks and made to the mark with ethanol. The concentrations of sulphamethoxazole solutions were

0.01%, 0.02%, 0.03%, 0.04%, 0.05%, and 0.06% $^{w}/_{v}$. Those of trimethoprim solutions were 0.002%, 0.004%, 0.006%, 0.008%, 0.010% and 0.012% $^{w}/_{v}$.

Solutions of drug samples

Twenty tablets each of Septrin (Wellcome) and Co-trimoxazole (Phyto-Riker) were separately weighed and powdered. A quantity of the powder (Cotrimoxazole (0.1477g) and Septrin (0.1485g)) equivalent to 100mg sulphamethoxazole and 20mg trimethoprim was weighed, dissolved in sufficient ethanol, transferred into 100ml volumetric flasks and the solutions shaken for about 15min. The solutions were then made to volume with ethanol and filtered. 40ml of the filtrate was transferred into a 100ml volumetric flask and made to the mark with ethanol such that the concentrations of sulphamethoxazole and trimethoprim were 0.04 and 0.008% $^{w}/_{v}$ respectively.

Internal Standard

Solutions of paracetamol, codeine phosphate, caffeine, salicylic acid and benzamide were prepared by weighing 0.10g of each and dissolving it in sufficient ethanol in a 100ml volumetric flask. The solution was then made up to the mark. The concentration of the resulting solutions was 0.1% $^{w}/_{v}$.

Mobile Phase

The ammonium perchlorate buffer was prepared by mixing 3.7ml of ammonia solution with 4.2ml perchloric acid (70%) in a 1000ml volumetric flask and made to the mark with distilled water. The pH of the solution was adjusted to 3.0 with 0.1M hydrochloric acid. The mobile phase was prepared by measuring separately the volume of each component and mixing them together. It was then filtered through a cellulose membrane filter before use.

Procedure for Analysis

Calibration graphs and assay of drug samples

Each of the calibration solutions (50ml) was thoroughly mixed with 25ml of internal standard solution and made up to 100ml with distilled water. Aliquots of 20ml of the mixture were successively introduced onto the hplc column for elution. The respective peak areas of chromatograms were then determined. Three replicate determinations of each concentration unit were done. Mean peak area ratios (MPAR) (peak area of drug sample/ peak area of internal standard) were calculated for both sulphamethoxazole and trimethoprim in each concentration unit analysed. A graph of mean peak area ratio against concentration of either sulphamethoxazole or trimethoprim was plotted to relate peak area ratios to concentrations.

For the assay of drug samples (Septrin and Co-trimoxazole), sample preparation, handling and analyses were the same as above. Actual concentration of either sulphamethoxazole or trimethoprim in a sample was interpolated from the required calibration graph using the respective MPAR.

Statistical analysis

Linear regression analysis was used for plotting calibration curves.

RESULTS AND DISCUSSION

Sulphamethoxazole and trimethoprim are compounds of moderate polarity (Moffat, 1986; BP 2002). This therefore required a reverse phase column with a polar mobile phase. In this mode of chromatography, solutes are separated according to their level of affinity for either the stationary phase or the mobile phase. Relatively more polar solutes are eluted first and the less polar last because of greater affinity for the stationary phase. Figure 2 therefore is a typical chromatogram where sulphamethoxazole, trimethoprim and salicylic acid showed distinct separation profiles as a result of differences in physicochemical properties. Under the conditions of chromatographic separation, trimethoprim was the most polar and salicylic acid the least polar with respective mean retention times of 3.4±0.13 min and 4.5±0.13 min. Sulphamethoxazole had

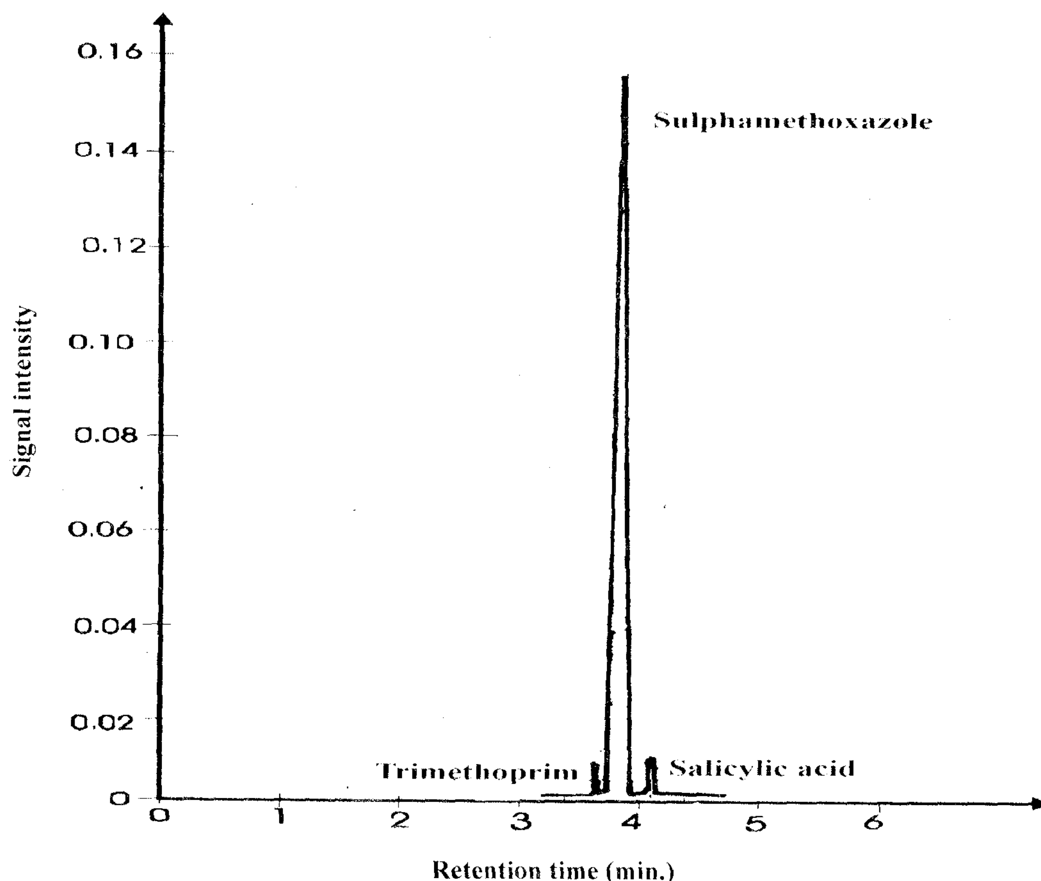


Fig. 2: Typical chromatogram of trimethoprim, sulphamethoxazole and salicylic acid (Internal standard)

an intermediate polarity with a retention time of 3.8±0.08 min. Other factors that could have affected the elution profile as well were the pH of the mobile phase and the respective acid dissociation constants (pka) of the drug samples.

The elaborate chromophore system coupled with auxochromes in both sulphamethoxazole and trimethoprim (Fig. 1) made it possible to monitor the eluate via UV absorption.

The overall pH of the mobile phase (3.0) also affected the wavelength of detection (254 nm) since the drug molecules had different absorption patterns in acidic, basic and neutral environments (Mofatt, 1986).

As shown in Table 1, the specific absorbance of both sulphamethoxazole and trimethoprim were high enough for minute quantities to be detected in the column effluent for quantitative purposes. Various methods including spectroscopy, fluorimetry, polarography and hplc have been used to analyse antimicrobial drugs. These methods require not only derivatization but also elaborate sample preparation and expensive equipment (Caturla *et al.*, 1992; Soltes, 1999). In the case

Table 1: Data for UV identification of drug

Sample	Concentration (%$^w/_v$)		Mean Absorbance		Mean Specific Absorbance	
	SMX	TRM	SMX	TRM	SMX	TRM.
Co-trimoxazole	0.0004	0.0005	0.2704	0.1081	676	250.2
Septrin	0.0004	0.0010	0.2696	0.2180	674	250.0
Pure powders	0.0004	0.0005	0.2688	0.1079	672	250.8

SMX: Sulphamethoxazole (λ_{max} 256 nm); TRM: Trimethoprim (λ_{max} 271 nm); Solvent: aqueous alkali

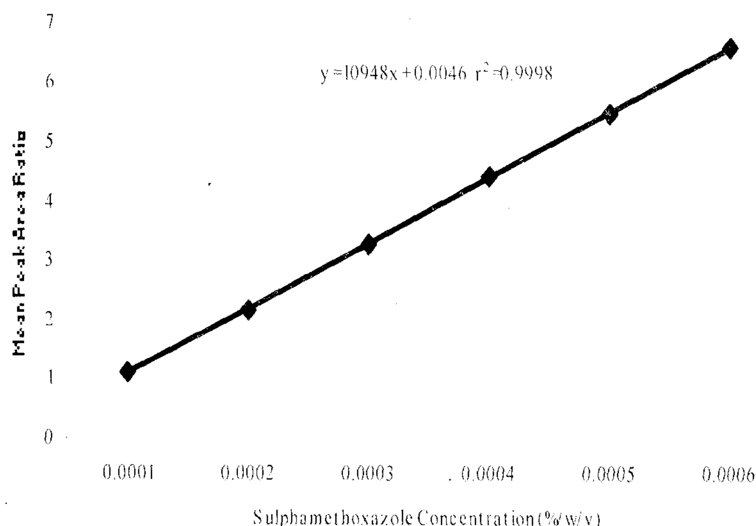


Fig. 3: Calibration graph of pure sulphamethoxazole for relating peak area ratios to concentration

of multicomponent antibiotic formulations, there can be interaction of the drugs (Aghazadeh and Kazemifard, 2001). The designed method under discussion is devoid of derivatization nor an elaborate sample preparation. Also, the mobile phase (ethanol (96%)/0.1M ammonium perchlorate buffer (95:5) is readily available and less expensive. It can be seen from Figures 3 and 4 that there was a linear correlation between peak area ratio and concentration of either sulphamethoxazole or trimethoprim. The sulphamethoxazole scatter had a co-efficient of correlation (r^2) of 0.9998 while that of trimethoprim was 0.9982 (Fig.3 and Fig.4). This meant that chromatographic response in terms of peak area ratio can quantitatively be related to concentration for the purposes of assays. The limit of detection was 0.01mg/ml for trime-thoprim and 0.00005 mg/ml for sulphamethoxazole. This was mostly because sulphamethoxazole in a similar chemical environment (aqueous alkali) had a higher absorptivity than trime-thoprim (Table 1). Figure

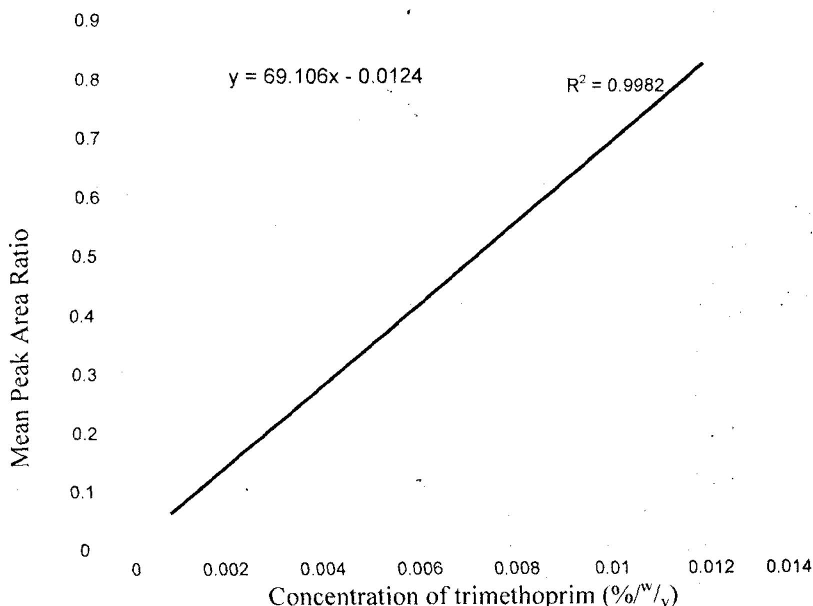


Fig. 4: Calibration graph of pure trimethoprim for relating peak area ratios to concentrations

2 actively suggests that the method was selective for sulphamethoxazole, trimethoprim and salicylic acid (internal standard). There was a clear separation without overlapping bands. Additionally, chromatograms seemed symmetrical enough to indicate complete resolution without tailing.

The new rp-lc method was further challenged by applying the procedure to assay two brands of co-trimoxazole tablets (Co-trimoxazole (Phyto-Riker) and Septrin (Wellcome)). Before the assays, both pure powders and drug products were characterized using the monograph specifications of the British Pharmacopoeia (2002). Such preliminary tests as colour identification, melting point, TLC profiles, uniformity of weight, friability and tablet hardness were carried out for drug sample characterization. As indicated in Table 2, both drug samples and pure powders (sulphamethoxazole and trimethoprim) complied with the monograph requirements of the tests carried out. These therefore suggest the authenticity of using the septrin and co-trimoxazole tablets to test the new analytical method for reproducibility and accuracy.

Assay of the contents of the two different brands by hplc yielded values that were consistent with BP (2002) monograph requirement for co-trimoxazole tablets. By this requirement, every co-trimoxazole tablet should contain 92.5-107.5% of the stated respective amounts of trimethoprim and sulphamethoxazole. It was realized from Table 3 that both brands of tablets had percentage contents of components within the stated range, suggesting the usefulness of the new rp-lc method for its purpose.

Rønn *et al.*, (1999), used rp-lc for the simultaneous analysis of trimethoprim, sulphamethoxazole and acetylsulphamethoxazole in small amounts of blood. The mobile phase consisted of 20% acetonitrile and 80% phosphate buffer adjusted to pH 6.15. Retention times of 3.9 min,

Table 2: Pharmacopoeial tests results

Sample	Colour tests	Melting Point (°C)		TLC Rf values		Uni-formity of weight	Friabil-ity	Tablet hardness/ Kg	% Purity	
		SMX	TRM.	SMX.	TRM				SMX	TRM.
Septrin	Passed	168-170	198-201	0.72	0.59	No deviation	passed	11.10	N/A	N/A
Co-trimoxazole	Passed	169-172	198-200	0.73	0.61	No deviation	passed	11.18	N/A	N/A
Pure powder	N/A	168-171	199-201	0.72	0.60	N/A	N/A	N/A	100.1	100.3

SMX: Sulphamethoxazole; TRM: Trimethoprim; N/A: Not applicable

Table 3: Rp-lc data for the assay of Septrin and Co-trimoxazole

	MPAR		Content (mg)		% Content	
Sample	SMX	TRM	SMX	TRM	SMX	TRM
Co-trimoxazole	1.00	0.96	160.8	30.72	100.0	96.0
Septrin	1.02	0.97	163.2	31.04	102.0	97.0

SMX: Sulphamethoxazole; TRM: Trimethoprim; MPAR: Mean Peak Area Ratio

5.7 min and 6.6 min were observed for acetylsulphamethoxazole, trimethoprim and sulphamethoxazole respectively. This new analytical method also had 3.4 min, 3.8 min and 4.5 min as retention times respectively for trimethoprim, sulphamethoxazole and salicylic acid. Apart from the high cost of acetonitrile in the previous work for routine analysis, the running time for the latter method is better than the former. Considering only the trimethoprim and sulphamethoxazole components of the analyses, it was realized that the former method had a total running time of approximately 7 min while the latter had 4 min, making it relatively more suitable for both in-process and finished product quality monitoring. However, both methods revealed the same order of polarity for both trimethoprim and sulphamethoxazole, trimethoprim being relatively more polar by reason of its shorter retention time.

CONCLUSION

An rp-lc method has been developed which analyses simultaneously the two components of Co-trimoxazole tablets devoid of the laborious extraction process of the official method. Separation was achieved with a mobile phase combination of 96% ethanol and 0.1M ammonium perchlorate buffer (95:5) on a reversed phase column at ambient temperature. Detection was by UV at a wavelength of 254 nm.

REFERENCES

Aghazadeh, A and Kazemifard, G. (2001). J. *Pharm. Biomed. Anal.*, 25, 325

Altun, M.L., and Erk, N. (2001). The rapid quantitative analysis of phenprobamate and acetaminophen by rp-lc and compensation technique. *Journal of Pharmaceutical and Biomedical Analysis. ABR*; 25 (1): 85-92

British National Formulary (March, 2003). BMA and RPSGB publications, *Internet; Http/L briandeer.com./Septrin/Bactrim-bnf.htm. July, 2005.*

British Pharmacopoeia (BP) (2002). Volumes I & II, CD-ROM, The British Pharmacopoeial Commission

Carturla, M. C., Cusido, E. and Westerlund, D. (1992). *J. Chromatogr.* 593, 69

Erk, N.and Altun, M.L. (2001). Spectrophotometric resolution of metronidazole and miconazole nitrate in ovules using ratio spectra derivative spectrophotometry and RP-LC. *Journal of Pharmaceutical and Biomedical Analysis.* ABR; 25 (1): 115-122

Ghana National Drugs Policy (GNDP) (1999). Ministry of Health, Ghana. pp 4,7,12 and 19

Moffat, A C. (1986). Clark's Isolation and Identification of Drugs in Pharmaceuticals, 2nd edition. London: The Pharmaceutical Press,

Rønn, A. M., Mutabingwa, T. K., Kreisby, S., Angelo, H. R., Fuursted, K. and Bygberg, I. C. (1999). A Reversed-Phase High-Performance Liquid Chromatography Method for the Determination of Co-trimoxazole (Trimethoprim/Sulphamethoxazole) in Children treated for malaria, *Ther Drug Monit,* 21(6), 609

Soltes, L (1999). Biomed. *Chromatogr.* 13, 3

World Health Organization Drug Information (1999). 13 (3)