Mitochondrial membrane preparation for MAO from rat brains :

The brains were homogenized in 10 ml of ice-cold 0.25 M sucrose, adijusted to pH 7.4 and centrifuged at 8000 x G for 15 min . The supernatents were recentrifuged at 20,000 x G for 30 min and the pellets were then homogenized in 0.01 M ice-cold phosphate buffer (pH 7.4) and centrifuged at 30,000 x G for 30 min for isolation of the mitochondrial fractions . Each pellet was then suspended in the same buffer and the protein concentration was adjusted to 10-12 mg protein/ml . They were stored at -20° C . The MAO activity was measured according to a reported method (16) in which p-nitrophenylethylamine hydrochloride was used as substrate .

References :

1) R.J. Borgman, J.J. MePhillips, R.E. Stitzel, and I.J. Goodman, **J. Med. Chem.**, 16, 630 (1973) .

2) J. G. Cannon, B.J. Demopoulos, J.P. Long, J.R. Flynn, and F.M. Sharabi, ***ibid.***, 24, 238 (1981) .

3) W.F. Huffman, J.W. Wilson, **U.S. Patent** 4314944, 1982; **Chem. abstr.** 96, 181146c (1982) .

4) C. Kaiser, P.A. Dandridge, E. Garvey, K.E. Flaim, R.L. Zeid, and J.P. Hieble, **J. Med. Chem.**, **28.** 1803 (1985) .

5) R.J. Borgman, M.R. Baylor, J.J. McPhillips, and R.E. Stitzel ***ibid.***, 17, 427 (1974) .

6) J.G. Cannon, Z. Perez, J.P. Long, D.B. Rusterholz, Flynn, B. Costall, D.H. Fortune and R.J. Naylor, ***il*** 22. 901 (1979) .

7) M. Ilhan, J.P. Long, and J.G. Cannon, **Eur. J. Pl macol.**, 33, 13 (1975) .

8) J.Z. Ginos, J.M. Stevens, and D.E. Nichols, **J. M Chem.**, 22, 1323 (1979) .

9) J.G. cannon, J.P. O'Donnell, T. Lee, C.R. Hoppin, J Long, M. Ilhan, B. Costall, and R.J. Naylor, ***ibid,*** 1 1212 (1975) .

10) J.G. Jackson. and J. Kenner, **J. Chem. Soc.**, 16 (1928) .

11) J.G. Cannon, T. Lee, H.D. Goldman, B. Costal and R. Naylor, **J. Med. Chem.**, 20. 1111 (1977) .

12) N.E. Anden, A. Rubenson, K. Fuxe, and T. Hökfelt, **Pharm. Pharmacol.**, 19, 627 (1967) .

13) L.S. Goodman, and A. Gilman, "**The Pharmacological Basis of Therapeutics**". 7th ed. Macmillan, New York, Toronto, London, 1985, p. 108 .

14) F. Lecnard, A. Wajngurt, M. Klein and C.M. Smith, **J. Org. Chem.**, 26, 4062 (1961) .

15) C.C. Chang, **J. Neuropharmacol.**, 3,643 (1964) .

16) M.M. Abdel-Samed, Z.I. Akopyan, I.V. Viryovkina, A.A. Kulygina and V.Z. Gorkin, **Biochem. Pharmacol.**, 20, 2571 (1971) .

Received: September 21, 1988
Accepted for publication: October 5, 1988

Derivative Spectrophotometric Determination of Mixtures of Sex Hormones in Oily Injections.

FAWZY A. EL-Yazbi, MOHAMED A. KORANY, OMAYMA ABEDEL-RAZAK and MAHMOUD. A. EL-SAYED.

Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt.

Abstract □ **Methods are presented for the determination of two compoment mixtures namely oestradiol benzoate-progesterone and oestradiol valerate - testosterone enanthate in oily injections. The methods depend on the application of derivative and derivative-differential ultraviolet spectrophotometry to resolve the interference due to spectral overlapping and oily formulation matrix. The methods were applied to the determination of these hormones in synthetic mixtures and in oily preparations. The coefficient of variation was less than 2%.**

Keyphrases □ **sex hormones, two-component mixtures, derivative spectrophotometry, derivative-differential spectrophotometry, determination in oily injections.**

The direct spectrophotometric methods especially in case of multicomponent dosage forms are often complicated by interference

from the formulation matrix and spectral overlapping of other active ingredients. Such interference has been treated by many methods, including orthogonal polynomials (1), differential spectrophotometry (2,3), Fourier series (4) and least squares (5). These methods require special attention in selecting assay parameters and need several steps of calculations.

Ultraviolet derivative (6) and differential - derivative spectrophotometry (7,8) are relatively new techniques that offer good resolution of two overlapping spectra and elimination of matrix interference in the assay of many drugs. The literature reveal many applications of these techniques to the determination of single - component dosage form containing excipient or degradation products (9,10). Only few reports have dealt with the determination of multicomponent dosage forms (11).

A high pressure liquid chromatographic method was reported for the determination of a mixture of testosterone enanthate and oestradiol valerate (12). The official method (13) for the determination of oestradiol valerate in oily injection is the UV-difference spectrophotometry. It was reported (14) that this method can only be applied if the oestrogenic content of the injection exceeds 10 mg/ml. In a previous paper (15) we have applied the difference-derivative spectrophotometry for the determination of oestradiol esters or testosterone and / or progesterone in oily injections.

This paper deals with an application of derivative and difference-derivative spectrophotometry for the assay of two-component mixtures namely, oestradiol benzoate - progesterone and oestradiol valerate - testosterone enanthate in pure forms and oily injections.

EXPERIMENTAL

Materials: All reagents and materials used were analytical grade. Standard solutions were prepared by dissolving 50 mg oestradiol benzoate or 50 mg progesterone in 100 ml methanol and 40 mg oestradiol valerate in 100 ml p-dioxane and 65 mg testosterone enanthate in 50 ml p-dioxane. Methanolic/KOH solution was prepared by dissolving 17.5 g of KOH in 100 ml methanol. Methanolic/HCl solution was prepared by diluting 8.3 ml of HCl to 1 litre with methanol (13).

Apparatus: A perkin-Elmer model 550S UV-VIS spectrophotometer with a fixed slit width (2 nm) and a Hitachi model 561 recorder were used. The spectra of test and reference solutions were recorded in 1 cm quartz cells over the range 340 to 220 nm. Suitable settings are: scan speed 120 nm/min; chart speed 60 nm/min; ordinate maximum and minimum was adjusted according to the magnitude of $D_2 = d^2 A/d \lambda^2$ values: response time 10 sec.

Procedures:

Calibration graphs:

Oestradiol benzoate-progesterone mixture:

For oestradiol benzoate: A 4 ml portion of the standard hormone solution was transfered into a 100 ml volumetric flask and diluted to 50 ml with methanol. 5 ml aliquot of IN aqueous NaOH solution was added. A blank solution was made by mixing 50 ml of methanol and 5 ml of IN aqueous NaOH solution in a 100 ml volumetric flask. The two flasks were heated in a boiling water-bath for 90 min. (using air condenser, compensating the volatilized methanol if necessary), cooled, neutralized with IN HCl solution and completed to volume with methanol. Accurately two equal portions of the hydrolysed solution (4-8 ml at 1 ml step) were transferred into two sets of 10-ml volumetric flasks (a and b) both sets were completed to 8 ml with methanol. For flasks (a) the volume was completed with methanolic/HCl, while for flasks (b) with methanolic/KOH. The solutions were allowed to stand at room temperature for 15 min. and $\Delta D_2 = d(\Delta A)^2/d\lambda^2$ (peak-height) was measured at 304 nm ***(Fig. 1d).***

For progesterone: A 10 ml portion of standard solution was transferred into a 50-ml volumetric flask and completed to volume with methanol. Five serial dilutions were prepared by diluting 6-10 ml (1 ml step) to 100 ml methanol and D_2 value (peak-height) at 252 nm was measured ***(Fig. 1b).***

Oestradiol valerate-testosterone enanthate mixture:

For oestradiol valerate: two equal portions of the hormone solution (0.4-0.8 ml at 0.1 ml step) were transferred into two sets of 10-ml volumetric flasks (a,b). To both sets a 5 ml aliquot of p-dioxane was added. The solution was diluted to 8 ml with methanol. For flasks (a). the volume was completed with

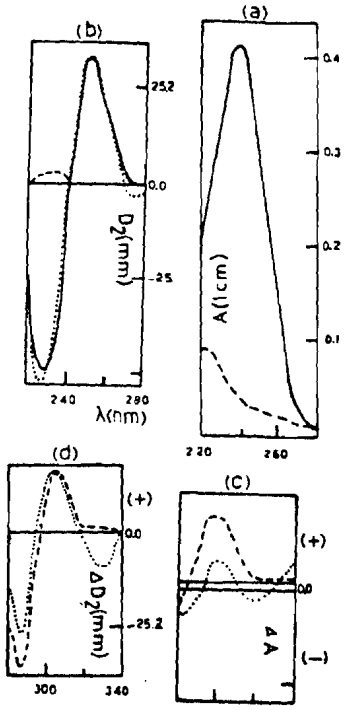

Figure 1: (a) absorption spectra of 0.8 mg% progesterone (_____) and 0.08 mg% oestradiol benzoate (– – –) (b) D_2 spectra of 0.8 mg% progesterone (_____), 0.08 mg% oestradiol benzoate (– – –) and oily injection (.....) containing the same concentration. (c) Δ A and (d) Δ D_2 spectra of 10 mg% progesterone (_____), 1 mg% hydrolysed oestradiol benzoate (– – –) and oily injection (.....) containing the same concentration.

methanolic/HCl while for flasks (b), with methanolic/KOH. The solutions were allowed to stand at room temperature for 15 min, and Δ D_2 values were measured at 266 nm (zero crossing point of testosterone enanthate) ***(Fig. 2b).***

For testosterone enanthate: A 5 ml portion of the hormone solution was transferredinto a 100-ml conical flask and a 5 ml aliquot of 1 N NaOH was added. About 40 ml of methanol was added and the content of the flask was heated on a water bath at 60 °C for 1 h. using air condenser (the volatilized methanol was compensated if necessary). After complete hydrolysis (as indicated by oil globules dissaperance) the alcohol was evaporated using a rotary evaporator and the residue was quantitatively transferred with about 20 ml water to a 100-ml separotory funnel. Testosterone was extracted with 20,20 and 5 ml portions of chloroform. The chloroform extract was passed over anhydrous sodium sulphate and received into a 50-ml volumetric flask and diluted to volume with chloroform. Five serial dilutions were prepared by diluting 7-11 ml (1 ml step) to 100 ml chloroform and D_2 (peak-height) at 250 nm was measured ***(Fig. 2e).***

Injection assay:

Oestradiol benzoate-progesterone mixture (1:10):

Sample preparation: An accurately measured volume of the oily injection (equivalent to 10 mg oestradiol benzoate and 100 mg progesterone) was transferredinto a 250-ml volumetric flask. A 100 ml aliquot of methanol was added and the solution was mechanically shaken for 15 min. The volume was completed with methanol and the solution was filtered through filter paper into a 250-ml volumetric flask.

For oestradiol benzoate: Exactly 50 ml of the filtrate was transferred into 100-ml volumetric flask, 5 ml IN aqueous NaOH solution was

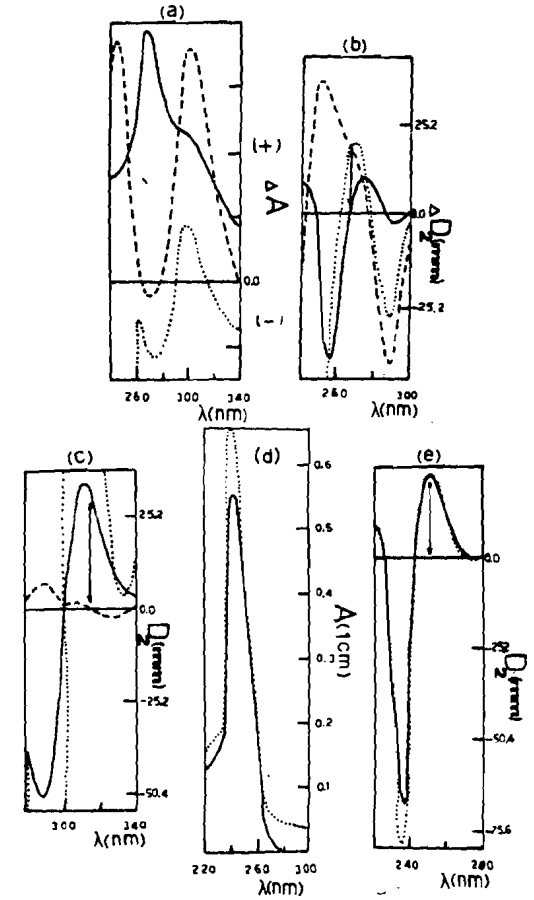

Figure 2: (a) Δ A and (b) Δ D_2 spectra of 52 mg%. T.E (_____), 3.2 mg% O.V (– – –) and oily injection (.....) containing the same concentration. (c) D_2 spectra of 1.04 mg% T.E (_____), 0.64 mg% O.V (– – –) and oily injection (.....) containing the same concentration. (d) absorption and (e) D_2 spectra of hydrolysed T.E (_____) and oily injection (.....) both equivalent to 1.264 mg% T.E. (T.E = testosterone enanthate, O.V = oestradiol valerate).

added and the procedure was completed as described under calibration graphs starting at the words "A blank solution"..

For progesterone: Five serial dilutions was prepared from the filtrate using methanol within the concentration range (0.6-1.0 mg%), and the D_2 (peak-height) at 252 nm was measured.

Oestradiol valerate-testosterone enanthate mixture (1:16.25):

Sample preparation: accurately 1 ml of the oily injection (equivalent to about 4 mg oestradiol valerate and 65 mg testosterone enanthate) was transferred into 50-ml volumetric flask and p-dioxane was added to volume.

For oestradiol valerate: The procedure was carried out as described under calibration graphs starting at the words "two equal portions of the hormone"..

For testosterone enanthate: Accurately 5 ml of the above solution was transferred into a conical flask, the procedure was completed as described under calibration graphs starting at the words "a 5 ml aliquot of 1N NaOH was added"..

RESULTS and DISCUSSION

Fig. (1a). shows that in 1/10th progesterone concentration, oestradiol benzoate exhibits broad absorbance band in the vicinity 240-260 nm ***(Fig. 1a)*** That is why the D_2 spectra of progesterone and oily injection are superimposable in such vicinity ***(Fig. 1b)*** indicating the nil contribution from oestradiol benzoate. In this connection, progesterone can be determined by measuring its D_2 values at 252 nm without interference from coexisting component. The minor component, oestradiol benzoate, was determined after hydrolysis to set free the phenolic group. Thereafter use of differential spectrophotometry permits selectively oestradiol assay. ***Fig. 1c and d*** presents the delta absorption (Δ A) and its second derivative (Δ D_2) spectra of progesterone and hydrolysed oestradiol as standard and oily injections. Inspite of the lack of pH-sensitive group, progesterone gives a constant Δ A versus λ curve ***(Fig. 1c).*** This may be due to the high progesterone concentration. Such contribution was completely corrected for by the use of Δ D_2 measurement ***(Fig. 1d). Fig. 2 a and b*** show the Δ A, Δ D_2 for oestradiol valerate and testosterone enanthate as standard and oily injection. Δ A curve ***(Fig. 2a)*** of about 16 parts of testosterone enanthate cover the vicinity 240 to 340 nm; a phenomenon which invalidate the use of Δ A measurement to quantitate the minor component (oestradiol valerate). Assay of such component can be made using Δ D_2 measurement at 266 nm ***(Fig. 2b)*** due to the zero-crossig of testosterone enanthate.

On the other hand, D_2 spectrum ***(Fig. 2c)*** of testosterone enanthate exhibites an appreciable D_2 value at 315 nm (zero-crossing point of oestradiol valerate). Meanwhile, interference from oil matrix, as indicated from ***(Fig. 2c),*** necessitated the prior extraction of testosterone during the injection assay. Through alkaline hydrolysis, the free testosterone can be extracted with chloroform. Being phenolic oestradiol valerate will remain with oil matrix in aqueous medium. After hydrolysis and extraction, the A_{max} method cannot be used for testosterone assay due to the background interference ***(Fig. 2d).*** Meanwhile the utility of D_2 method at 250 nm corrects for such interference ***(Fig. 2e).*** Under the described experimental conditions, a linear correlations were

Table I: Assay Results of Oestradiol Benzoate (OB) - Progesterone (PR) and Oestradiol Valerate (OV)-Testosterone Enanthate (TE) in Laboratory Made Mixtures Using the Proposed Methods.

Sample	Oestradiol benzoate-progesterone mixtures			Oestradiol valerate-testosterone enanthate mixtures		
	Ratio PR:OB	Recovery, %		Ratio OV:TE	Recovery, %	
		PR	OB		OV	TE
1	1:1	103.8	99.7	1:10	100.6	99.8
2	2:1	101.8	99.9	1:18	101.4	99.8
3	5:1	100.1	100.1	1:20	99.8	100.1
4	10:1	100.1	100.0	1:30	100.4	99.9
5	20:1	100.1	100.5			
6	40:1	100.4	100.5			
Mean ± C.V.%		101.1 ± 1.5	100.1 ± 0.3		100.8 ± 0.7	99.8 ± 0.2

obtained between D_2 and ΔD_2 and concentrations, C, of the hormone. The four linear regression equations were found to be $\Delta D_2 = -1.75 + 34.13\,C$ (range 0.8 – 1.6 mg%) for oestradiol benzoate, $D_2 = -1.55 + 83.26\,C$ (range 0.6 – 1.0 mg%) for progesterone, $\Delta D_2 = 0.75 + 12.76\,C$ (range 1.6 – 3.2 mg%) for oestradiol valerate and $D_2 = 0.15 + 16.95\,C$ (range 0.9 – 1.6 m%) for testosterone enanthate (where D_2 and ΔD_2 were measured in mm). The correlation coefficients were between 0.9979 and 0.9998.

Replicate determinations of the standard hormone solution (5 separate determinations) using the proposed methods gave a coefficient of variation less than 2%, indicating good reproducibility of the proposed methods. 5 synthetic mixtures of each combination were prepared in different proportions of hormone components and analyzed using the above procedures. The results obtained ***(Table I)*** were both precise and accurate.

The present methods were applied for the determination of these hormone combination in their commercial oily injections. The results obtained for the analysis of Primodian depot^R ampoules (Cid/Schering Co., Egypt), labelled to contain 4 mg oestradiol valerate and 65 mg of testosterone enanthate per 1 ml, were 100.2% ± 0.89 and 105.3% ± 0.52 (n = 5) for the two hormones respectively and for Lutofolone^R ampoules (Misr Co., Egypt). labelled to contain 2 mg of oestradiol benzoate and 20 mg of progesterone per 1 ml, were 100.3% ± 0.17 and 100.6% ± 0.33 (n = 5) for the two hormones respectively. In the oestradiol valerate-testosterone enanthate mixture, the D_2 measurement at 315 nm was applied directly for testosterone assay in laboratory made mixtures. In oily injection prior extraction and subsequent D_2 measurement at 250 nm solved the problem of oily matrix interference.

References:

1) H. Abdine, A.M. Wahbi, M.A. Korany, **J. Pharm Pharmac.**, 23, 444 (1971).
2) G. Aulin-Erdtman, **Chem. Ind.**, 74, 581 (1955).
3) A.M. Wahbi and A.M. Farghally, **J. Pharm. Pahrmac.**, 22, 848 (1970).
4) A.M. Wahbi, H. Abdine and M.A. Korany, **Pharmazie**, 33, 278 (1978).
5) B.W. Madsen, D. Herbison-Evans and J.S. Roberston, **J. Pharm Pharmac.**, 26, 629 (1974).
6) A.M. Wahbi and S. Ebel, **Anal. Chim. Acta.**, 70, 57 (1974).
7) M.A. Korany, F.A. El-Yazbi, O. Abdel-Razak and M.A. Elsayed, **Pharmaceutisch Weeklad**, 7, 163 (1985).
8) F.A. El-Yazbi, M.A. Korany, and M. Bedair, **J. Pharm. Belg.**, 40, 244 (1985).
9) M.A. Korany, A.M. Wahbi, M.A. Elsayed and S. Mandour, **Il Farmaco, Ed. Pr.**, 39, 243 (1984).
10) F.A. El-Yazbi, M.H. Barary and M.H. Abdel-Hay., **Int. J. Pharmaceutics**, 27, 139 (1985).
11) M.A. Korany, A.M. Wahbi, S. Mandour and M.A. Elsayed, **Anal. Letters**, 18 (1B), 21 (1985).
12) G. Carignan, B. Lodge and W. Skakum, **J. Chromatogr.**, 301, 292 (1984).
13) **"United State Pharmacopeia XIX"** USP convention INc., Rockville. Md., p. 181 (1975).
14) T. James, **J. Assoc. Off. Anal. Chem.**, 54, 1192 (1971).
15) F.A. El-Yazbi, M.A. Korany, O. Abdel-Razak and M.A. Elsayed, **Anal. Letters**, 18 (B17), 2127 (1985).

Received: July 17, 1988 .
Accepted for publication: October 5, 1988

Potentiation of Rifampicin by Trimethoprim

GISELE G. TAWIL

Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt.

Abstract □ **The combined bacteriostatic activity of rifampicin and trimethoprim was antagonistic against *Staph. aureus*, and synergistic or additive against *E. coli*. The concentration of rifampicin in the synergistic ratios corresponds to its plasma peak level. The combined bactericidal activity against *E. coli* was synergistic at concentrations corresponding to urine peak level of rifampicin.**

Keyphrases □ **Combined rifampicin-trimethoprim, *Escherichia coli*, *Staphylococcus aureus*.**

In vitro **studies showed that rifampicin is bactericidal at fractions of one ug/ml on Gram-positive bacteria and *M. tuberculosis*** and at concentrations of the order of several ug/ml on most Gram-negative microorganisms (1,2). When used in combination with several other chemotherapeutic agents, rifampicin displays ***In vitro*** synergism against a wide variety of microorganisms e.g. with novobiocin against ***S. typhi*** (3); with nafcillin against ***staph. aureus*** (4); with nalidixic acid against various Gram-negative bacteria (5); withmetronidazole against ***Bacteroides fragilis*** (6); with amphotericin against ***Candida albicans*** (7);

with trimethoprim against Proteus spp., ***Streptococci*** and ***Ps. aeruginosa*** (2). However, with some other organisms, the results of rifampicin-trimethoprim combinations were controversial, antagonism was reported against ***K. aerogenes*** and ***Strept faecalis*** (8), while synergism or additive effect occurred against ***K. aerogenes,*** and synergism against ***Strept. faecalis*** (2). The aim of this work was to evaluate the combined bacteriostatic and bactericidal activities of rifampicin and trimethoprim against two standard strains of ***Staph. aureus*** and ***E. coli.*** Since the minimum inhibitory concentrations of the tested agents may vary with the type of medium used, and hence influence the results, two media (nutrient broth and Mueller-Hinton broth) were used for comparison.

EXPERIMENTAL

Antimicrobial agents: Rifampicin (B.P.), Trimethoprim (B.P.)

Test organisms: Escherichia coli NCTC 10418 ***Staphylococcus aureus*** ATCC 25921.

Culture media: Nutrient Broth, Nutrient Agar, Mueller-Hinton Broth, Mueller-Hinton Agar "OXOID"

Methods:

A- Evaluation of the combined bacteriostatic activity: Stock solutions of rifampicin (RF) and trimethoprim (TMP) were prepared by dissolving 100 mg of Rf in 10 ml dimethylsulfoxide and then diluted to 100 ml with distilled water. Similarly TMP was dissolved in 10 ml alcohol and diluted with water to 100 ml. The final concentration of each drug was 100 µg/ml. The minimum inhibitory concentration of each drug was estimated by the twofold serial dilution method using nutrient broth and Mueller-Hinton broth against ***E. coli*** and ***Staph. aureus*** separately. The final inoculum size was $1x10^5$ cells/ml. The results were read after 24 hr incubation at 37 °C.

Solutions containing four times the MIC of each drug were prepared, and these were used to prepare combinations of the two drugs at different ratios ***(Tables I & II).*** The MIC values of the tested drugs singly and in combination were evaluated by the serial dilution method in nutrient broth and Mueller-Hinton broth against two tested microorganisms. In order to find out whether the combined activity was synergistic, antagonistic or additive, the partial concentrations of each component in the bacteriostatic mixture was calculated. This was done by dividing the concentration of each drug in the mixture by its individual MIC. The resulting ratios were expressed as fractional inhibitory concentrations (FIC). The sum

Table I: Combined Bacteriostatic Activity of TMP and RF Against ***E. coli*** Using Mueller-Hinton Broth and Nutrient Broth.

Ratio TMP:RF	MIC (µg/ml) TMP + RF	FIC TMP + RF	FIC index	Activity index
MHB				
100:0	0.780 + 0	1 + 0	1	0
80:20	0.624 + 5	0.799 + 0.1	0.899	-0.0462
60:40	0.234 + 5	0.3 + 0.1	0.4	-0.3972
40:60	0.312 + 15	0.4 + 0.3	0.7	-0.1549
20:80	0.156 + 20	0.2 + 0.4	0.6	-0.2218
0:100	0 + 50	0 + 1	1	0
NB				
100:0	50 + 0	1 + 0	1	0
80:20	25 + 5	0.5 + 0.1	0.6	-0.2218
60:40	15 + 10	0.3 + 0.2	0.5	-0.3010
40:60	20 + 30	0.4 + 0.6	1.0	0
20:80	10 + 40	0.2 + 0.8	1.0	0
0:100	0 + 50	0 + 1	1	0

Table II: Combined Bacteriostatic Activity of TMP and RF Against ***Staph. aureus*** Using Mueller-Hinton Broth and Nutrient Broth.

Ratio TMP:RF	MIC (µg/ml) TMP + RF	FIC TMP + RF	FIC index	Activity index
MHB				
100:0	0.78 + 0	1 + 0	1	0
80:20	1.247 + 0.003	1.599 + 0.2	1.799	0.2529
60:40	0.936 + 0.006	1.2 + 0.4	1.6	0.2041
40:60	0.624 + 0.009	0.8 + 0.6	1.4	0.1461
20:80	0.312 + 0.012	0.4 + 0.8	1.2	0.0792
0:100	0 + 0.015	0 + 1	1	0
NB				
100:0	12.5 + 0	1 + 0	1	0
80:20	10 + 0.002	0,8 + 0.025	0.825	-0.0835
60:40	30 + 0.016	2.4 + 0.2	2.6	0.4150
40:60	40 + 0.048	3.2 + 0.6	3.8	0.5798
20:80	10 + 0.032	0.8 + 0.4	1.2	0.079
0:100	0 + 0.08	0 + 1	1	0