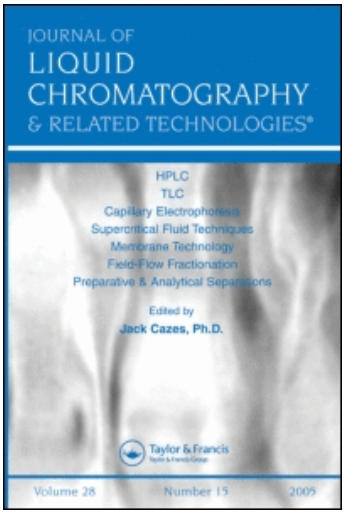

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APPLICATION OF CENTRAL COMPOSITE DESIGN TO THE OPTIMIZATION OF HPLC ANALYSIS OF NITROIMIDAZOLES

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Abstract

A 2 factor central composite design is used to study the effects of acetonitrile concentration and eluent pH on the retention and resolution of metronidazole and tinidazole on a C-18 column. The equations obtained have been used to predict the composition of an eluent for an optimum separation. The assay developed has been applied to the quantitation of metronidazole in human plasma following simple protein precipitation. The detection limit at 320nm for metronidazole is 0.01μg/mL. Using this assay a relative bioavailability study of two commercial metronidazole tablets in ten healthy volunteers has been performed.

Introduction

Nitroimidazoles such as metronidazole and tinidazole are agents used in the treatment of anaerobic infections and protozoal infestations. A number of methods for assaying these compounds have been reported(1-8). However these have all been developed

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empirically. In this investigation, a modified form of the factorial design known as a central composite design was used to determine the optimal experimental conditions for assaying these compounds. The assay developed has been applied to a relative bioavailability study of two commercial forms of metronidazole tablets.

Experimental

Apparatus and Materials

The Chromatographic system consisted of an LKB 2150 HPLC pump (Stockholm, Sweden), and a Shimadzu model SPD-6A variable wavelength detector (Kyoto, Japan). Samples were introduced by either a Rheodyne 7125 injector (Cotati, CA, USA) with a 20μL sample loop or a Waters 712 autoinjector (Milford, MA, USA). The stationary phase 5μm diameter ODS Hypersil (Shandon, London, UK) was packed in a 100 x 2mm id column by slurry techniques. Its efficiency was over 4000 plates per 10cm. Chromatograms were recorded on either a Rikadenki recorder or a Shimadzu CR3A (Kyoto, Japan) integrator. Metronidazole was obtained from Evans Medical Ltd (Palmerston North, New Zealand) and tinidazole from Sigma Chemical Co (St Louis, MO, USA). Sodium acetate,acetic acid, trichloroacetic acid, orthophosphoric acid and disodium hydrogen phosphate were purchased from BDH Chemicals (Poole, UK). Acetonitrile was purchased from J.T.Baker (Phillipsburg, NJ, USA). Water was double glass distilled and MilliQ® filtered. All reagents were of Analar grade or better. Generic metronidazole tablets were supplied by Evans Medical Ltd (Palmerston North, New Zealand) and Flagyl® tablets were from May & Baker Ltd (Wellington, New Zealand). Both dosage forms contained 400mg metronidazole.

Experimental Design

The traditional approach to assay development involves variation of one factor at a time while keeping the others constant. Such empirical techniques assume that there is no interaction between the various factors. In contrast, factorial designs allow evaluation of effects caused by independent variables as well as the interactions between them. In cases where the dependent variables do not vary linearly with the independent variables, factorial designs with at least three levels must be employed. However in many studies of this type, data obtained from using a three level factorial design may not be able to accurately locate the optimal experimental region. This can be seen by examining the layout of a 3 x 3 factorial design in Figure 1a. Eight of the nine experiments performed are on the boundary of the experimental region hence very little information is available within the central region. This will not pose many problems where the quadratic surface is nearly symmetrical about the centre. However an asymmetric parabolic response may not be adequately characterized. In such situations a modified factorial design known as a central composite design may yield better results. The layout of a 2 factor central composite design is shown in Figure 1b. The major difference between the two experi-

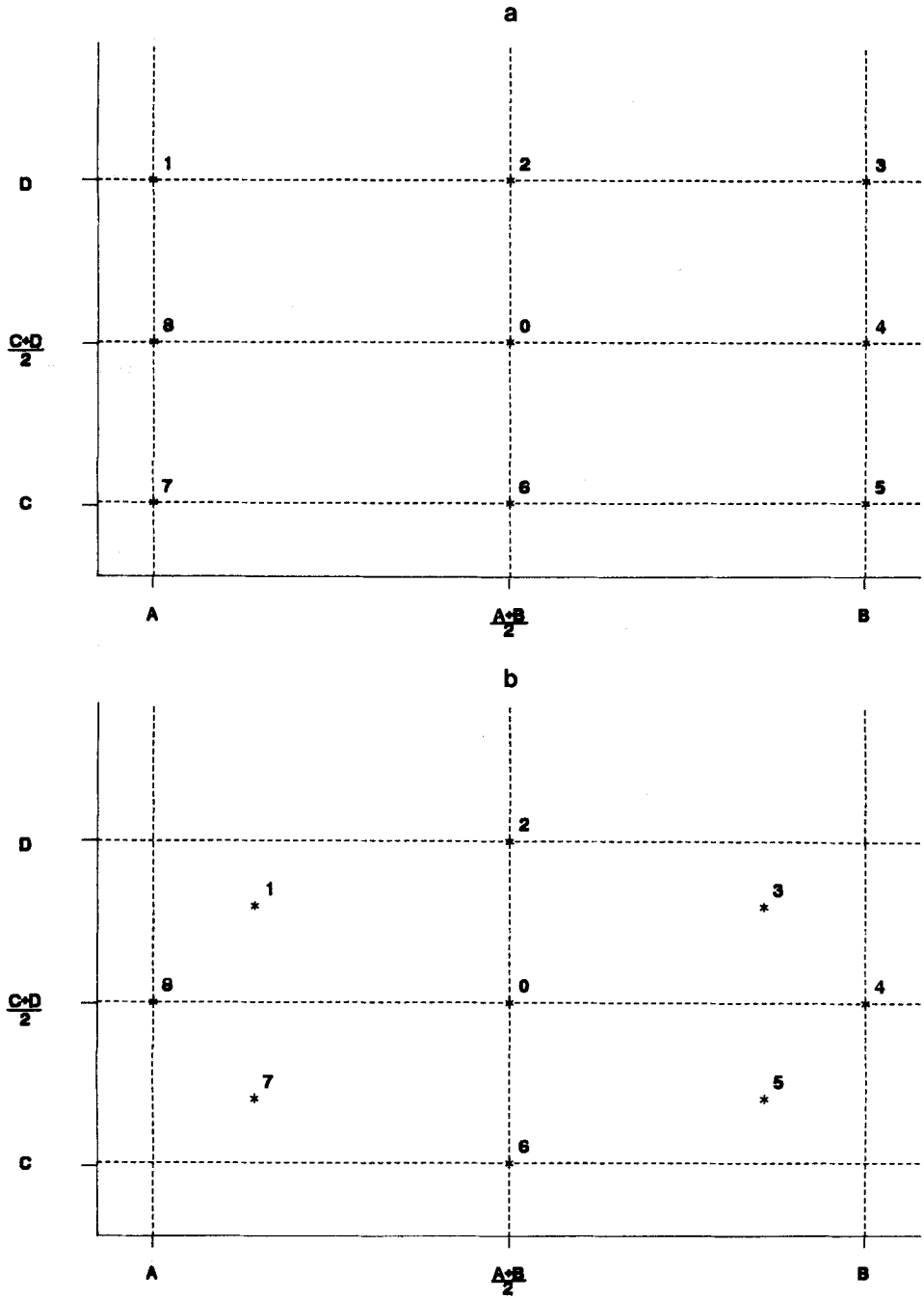

Figure 1. Layout of; (a) a 3x3 factorial design; (b) a 2 factor central composite design

mental designs is that the location of the four corner points 1,3,5 and 7 of Figure 1b are closer to the central point, point 0, than those of Figure 1a. Thus central composite design enables information to be collected from the boundary as well as from the centre of the experimental region. Furthermore the same number of experimental points can provide five levels of information with a two factor central composite design, compared to only three levels with a 3 x 3 factorial design. The details for setting up a central composite design have been discussed by Box and Draper(9). In this investigation , the central point (point 0) of the the experimental design was repeated twice to allow the "lack of fit" of the model to be estimated making a total of eleven experiments. Acetonitrile was chosen as the organic modifier because it exhibited a lower back pressure than methanol. The various levels used for acetonitrile concentration and pH are shown in Table 1. The buffer salt concentration was kept constant (20mM disodium hydrogen phosphate) and pH was adjusted to the desired value with orthophosphoric acid. All experiments were conducted at a constant temperature of $28 \pm 2°C$. Experiments were performed and data analyzed as previously described(10).

Metronidazole Bioavailability Study

Ten healthy male volunteers aged between 20 and 30 participated in a two-way cross-over bioavailability study. They were divided into two groups. The first group was given one 400mg Flagyl® tablet and the second group one 400mg generic metronidazole tablet. One week later the second group received the Flagyl® tablet and the first group the generic product. Blood samples were collected into heparinized tubes at predetermined times over a 24 hour period after drug administration. The samples were centrifuged and the plasma transferred into plastic containers and stored at $-15°C$ until analysis.

Table 1: Experimental levels in the central composite design

Point	pH	%ACN
$0,-\alpha$	4.5	0
-1,-1	3	2
+1,-1	6	2
$-\alpha,0$	2	7
0,0	4.5	7
-1,+1	3	12
+1,+1	6	12
$0,+\alpha$	4.5	14
$+\alpha,0$	7	7

Note: calculated values for pH and acetonitrile concentration were rounded to convenient values.

Sample Preparation

To 500μL of plasma in a 1.5mL plastic centrifuge tube 50μL of internal standard (68μg/mL aqueous tinidazole solution) was added. Proteins were precipitated using 150μL of 60%w/v aqueous trichloroacetic acid solution while vortexing. The sample was centrifuged at 8000xg for 30 minutes. The clear supernatant was then transferred to a small vial and 150μL of aqueous saturated sodium acetate added. 25μL of this solution was injected on to the column.

Chromatography

Analysis of the samples for metronidazole was performed using an eluent of acetonitrile water (5:95v/v) containing 20mM sodium acetate. The pH of the eluent was adjusted to 5 with acetic acid. Detection was at a wavelength of 320nm and eluent flow rate was set at 0.5mL/min.

Results and Discussion

Central Composite Design

There are a number of chromatographic variables that are important in achieving a separation. In this investigation the column temperature was held constant, as the variation of column temperature requires special apparatus(11). The eluent buffer salt concentration was fixed at 20mM because preliminary studies showed that it had minimal effect on the retention of metronidazole and tinidazole. On the other hand the organic modifier content and the pH of the mobile phase were found to have a marked effect on the retention and separation of the weakly basic nitroimidazoles (pKa≈2.5)(12). The observed k' of metronidazole and tinidazole in the eleven eluents as determined by the central composite design are presented in Table 2. Initial attempts to fit the log k' of the compounds as a function of the organic modifier content and pH of the mobile phase were unsucessful. None of the models derived passed the test for lack of fit(9) (p=0.002). The method of maximum likelihood(9) was then adopted for locating the most appropriate model. The models thus obtained for metronidazole and tinidazole are shown in equation 1 and 2 respectively.

$$k'(met) = \frac{1}{(.4275+.0511(\%ACN)-1.5067/pH+.0336(\%ACN)/pH-.0003(\%ACN)^2+3.0417/pH^2)^2} \quad (1)$$

$$k'(tin) = \frac{1}{(.2067+.036(\%ACN)-.5746/pH-1.1217/pH^2)^2} \quad (2)$$

The observed data fit adequately to the derived models with p values greater than 0.4. The inverse relationship between the k' of the compounds and the pH of the eluent is similar to that suggested by Salto et al(13). The plots of k' as a function of acetonitrile concentration and the pH of the mobile phase are presented in Figures 2a and b. The presence in equation 1 and 2 of an interaction term indicates that empirically optimized

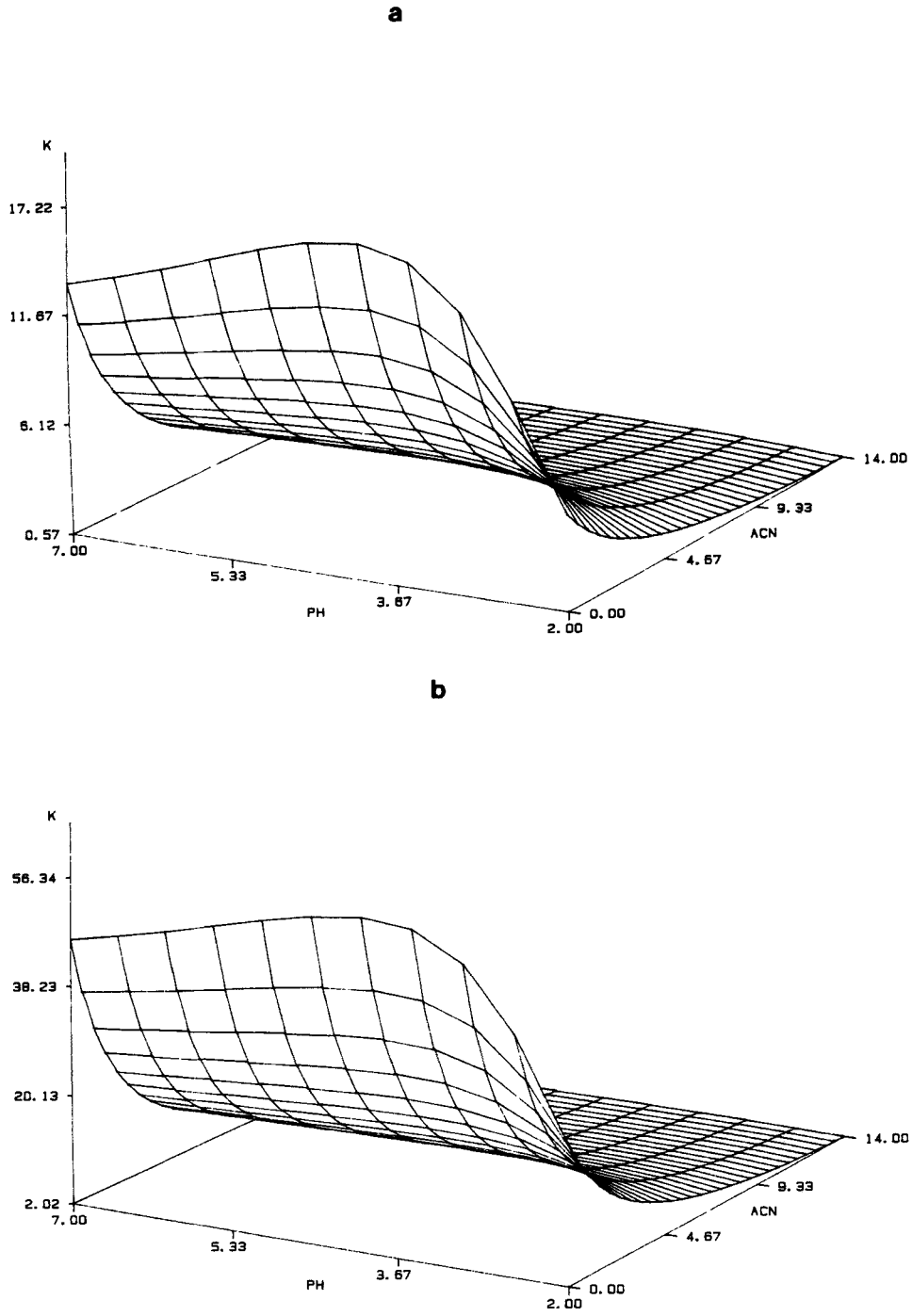

Figure 2. Capacity factor map for; (a) metronidazole; (b) tinidazole; see table 2 for chromatographic conditions

assays may have some difficulties in locating the optimal chromatographic condition. The contour plots of k' for metronidazole and tinidazole between 2.5 and 20 were generated using equations 1 and 2, and presented in Fiures 3a and b. The resolution plot (Figure 4) generated by using equation 3 reveals that resolution greater than 5 between the two compounds can be achieved throughout most of the experimental region.

$$Rs = \frac{\sqrt{N}}{4}\frac{\alpha-1}{\alpha}\frac{k'}{k'+1} \quad (3)$$

Thus appropriate retention time and stable chromatographic conditions are the sole criteria for selecting the mobile phase composition. Since nitroimidazoles can be monitored at 320nm with adequate sensitivity, chromatograms of the plasma extract recorded at this wavelength are relatively clean. Hence solutes with capacity factor of greater than 3 can usually be resolved from the plasma constituents. Examination of the contour plots for the two compounds (Figure 3a and b) indicates that retention of metronidazole with k' around 3 can be obtained when pH is in the range of 2 to 7 and with acetonitrile content from 0 to 7%v/v. However Figures 2a and b demonstrate that retention of metronidazole and tinidazole are most stable when pH is between 4 to 6 and acetonitrile concentration is between 4.5 to 7%v/v. For convenience the mobile phase pH was set at 5 and acetonitrile concentration at 5%v/v. Using this mobile phase capacity factors of 3.5 and 9.3 were obtained for metronidazole and tinidazole respectively. The predicted k' value for metronidazole was 3.57 ± 0.2 and for tinidazole 9.96 ± 1.1 (errors shown represent 95% confidence limits). This confirms the predictive power of the

Table 2: Experimental capacity factors of metronidazole and tinidazole

Mobile Phase composition		k'	
pH	%ACN	metronidazole	tinidazole
4.5	0	17.667	58.000
3	2	6.667	22.416
3	2	6.917	21.000
2	7	1.250	4.917
4.5	7	2.417	6.417
4.5	7	2.458	7.000
4.5	7	2.500	6.833
7	7	2.417	6.000
3	12	1.083	3.000
6	12	1.250	3.167
4.5	14	1.000	2.417

Chromatographic conditions: column 100 x 2mm ODS-Hypersil; flow rate 0.5ml/min; temperature $28 \pm 2°C$.

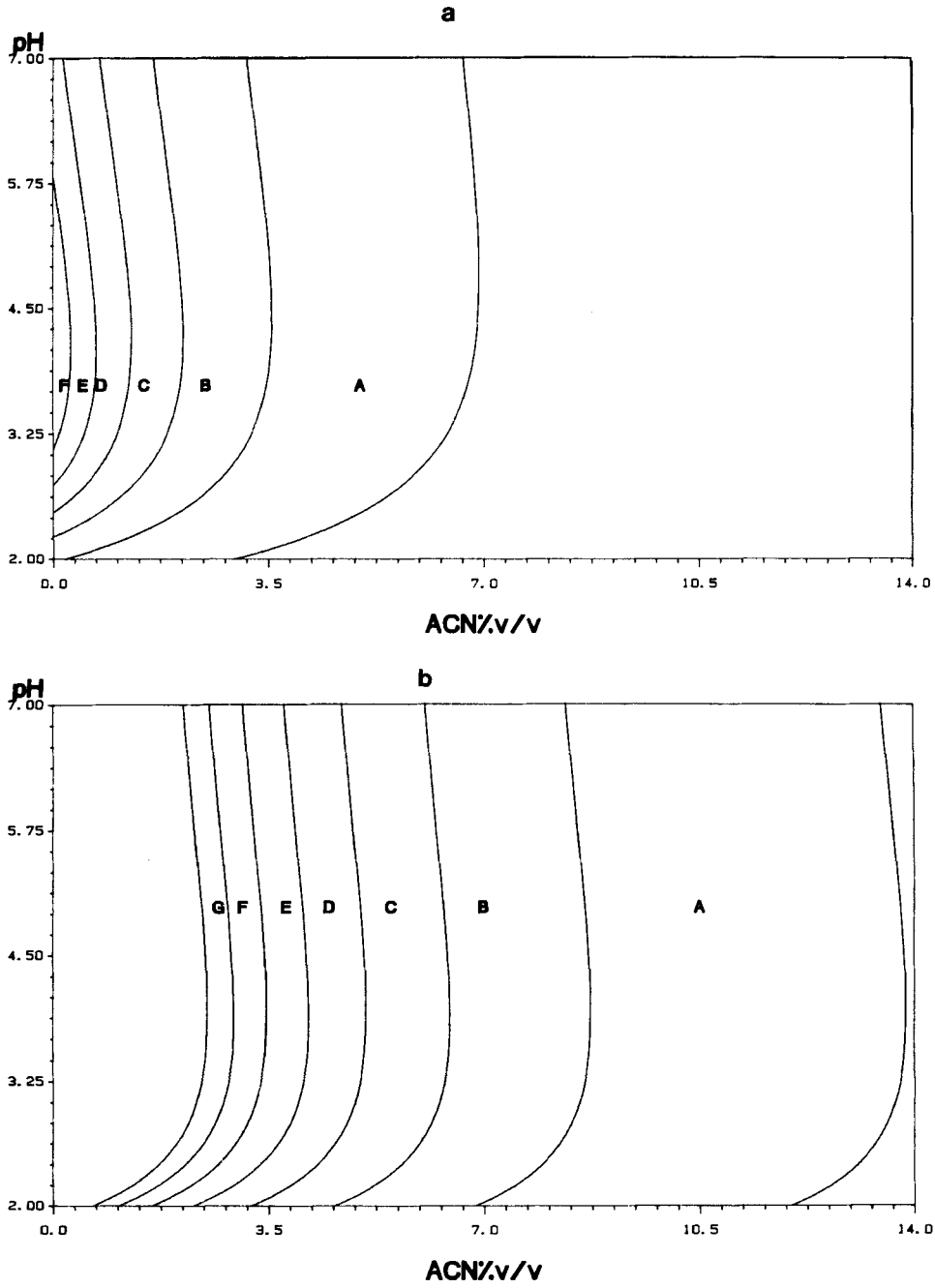

Figure 3. Experimental conditions that provide k' between 2.5 and 20 for; (a) metronidazole; (b) tinidazole
A=2.5-5, B=5-7.5, C=7.5-10, D=10-12.5, E=12.5-15, F=15-17.5, G=17.5-20

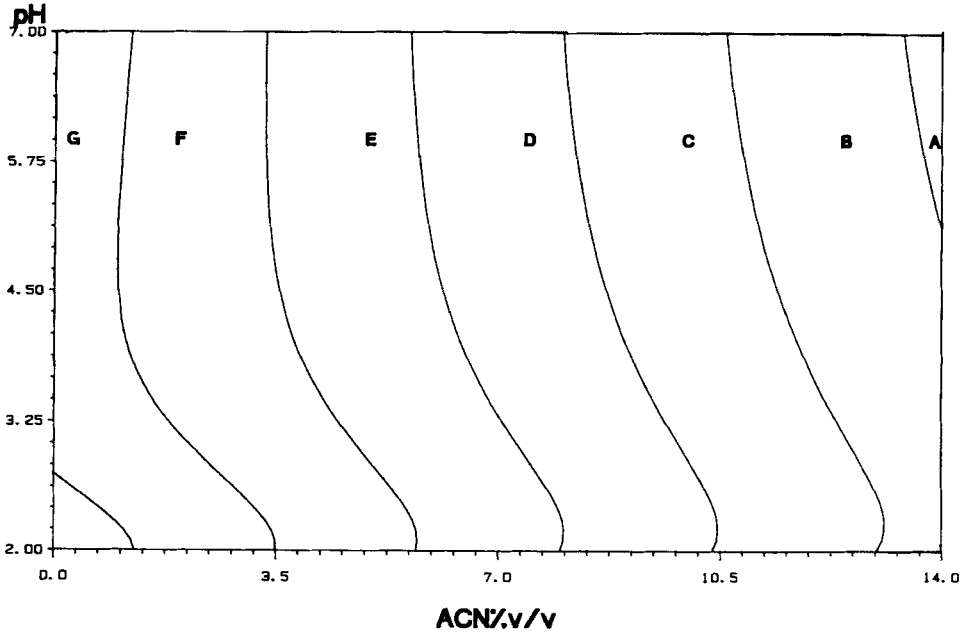

Figure 4. Resolution plot of metronidazole and tinidazole.
A=0-6.5 , B=6.5-7.5 , C=7.5-8.5 , D=8.5-9.5 , E=9.5-10.5, F=10.5-11.5, G=11.5-12.5

models. In view of the poor buffer capacity of phosphoric acid and its salt at pH5 (14), the buffer was replaced by 20mM sodium acetate and acetic acid. This substitution did not alter the retention characteristics of the solutes. A typical chromatogram is shown in Figure 5.

Sample Preparation

Direct injection of sample after protein precipitation was studied(15). Saturated aqueous ammonium sulphate solution and aqueous zinc sulphate/sodium hydroxide solutions proved to be ineffective protein precipitants. The use of 60%w/v perchloric acid was effective but produced poor peak shape due to the large decrease in the eluent pH. Attempts to neutralize the acid with sodium hydroxide were found to be tedious and non reproducible. In addition, it caused rapid deterioration of the column due to the disintegration of the silica gel at higher pH. Trichloroacetic acid 60%w/v similarly caused marked peak tailing. However addition of saturated aqueous solution of sodium acetate to the supernatant resulted in a pH around 5 and maintained the peak sharpness. This

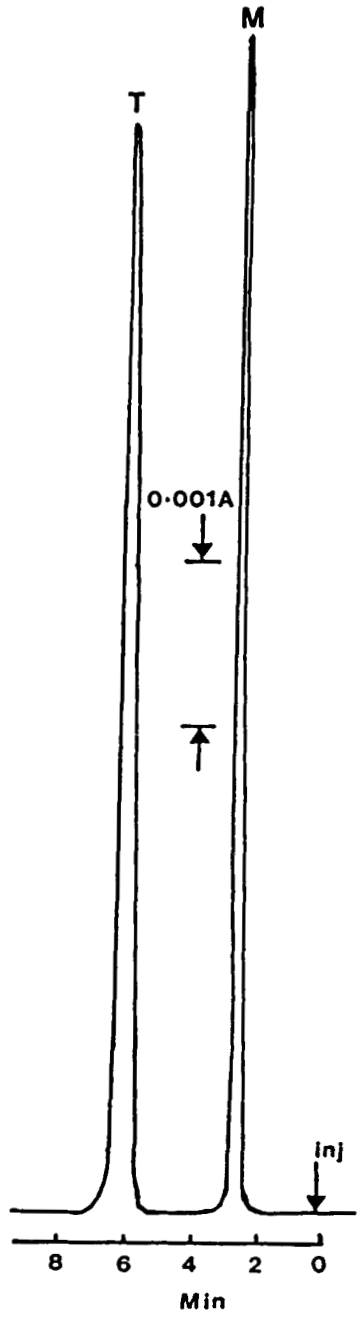

Figure 5. Chromatograhic seperation of the two nitroimidazoles. Peak identification: M (250μg/mL metronidazole), T (250μg/mL tinidazole), see text for chromatographic conditions.

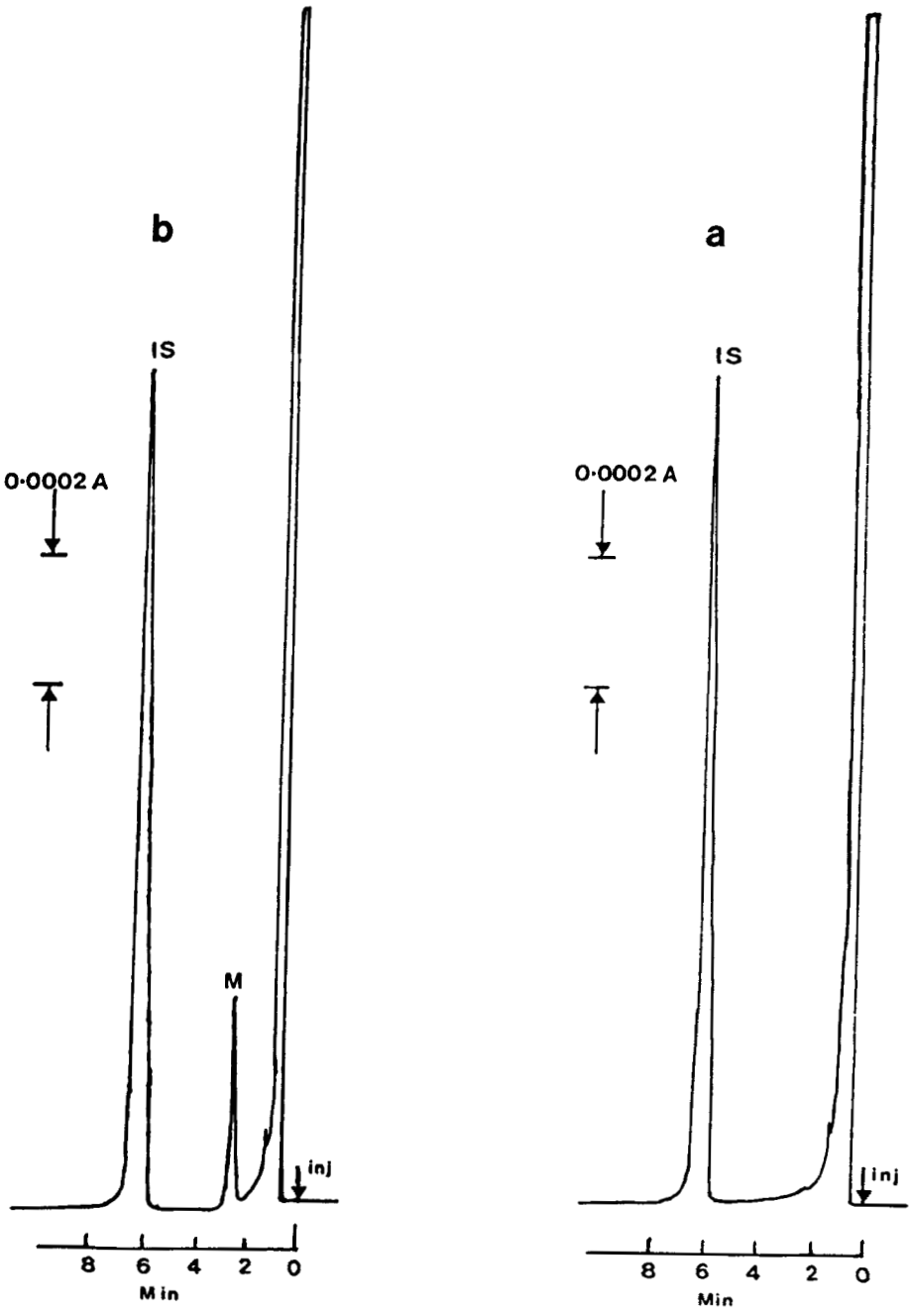

Figure 6. Chromatographic separation of two nitroimidazoles in plasma, injection volume 25μL; (a) blank plasma sample; (b) plasma spiked with known quantity of metronidazole. Peak identification: M metronidazole (0.5μg/mL); IS tinidazole (68μg/mL); injection volume

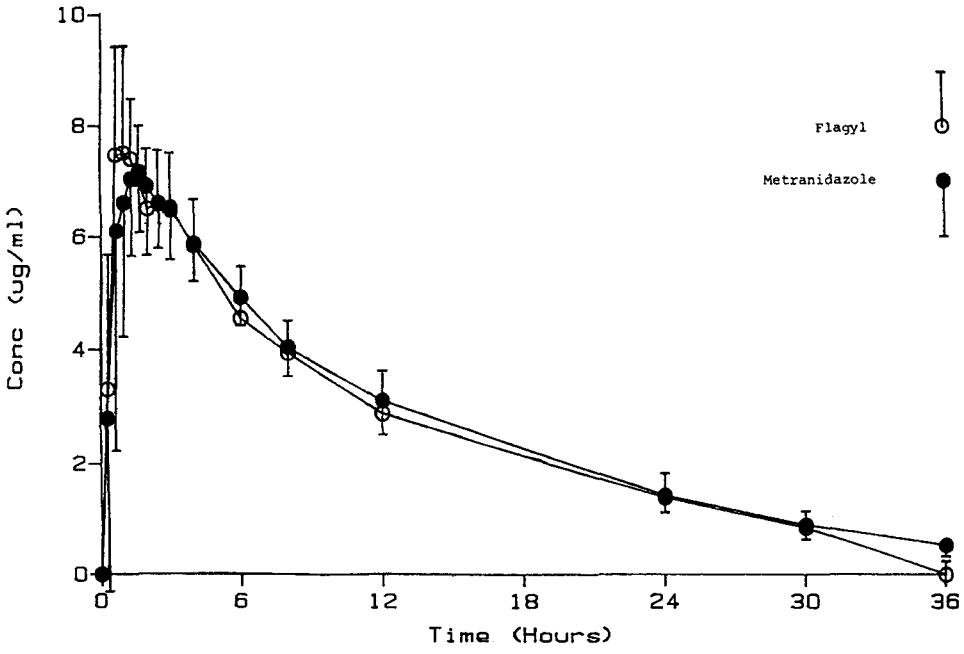

Figure 7. Mean plasma metronidazole concentration resulting from the oral administration of one 400mg Flagyl® tablet and one 400mg generic metronidazole tablet.

method was adopted and used successfully for more than 300 plasma samples. Figure 6 shows the chromatograms of a subject blank plasma sample and human plasma with a known concentration of metronidazole and internal standard tinidazole.

Linearity Sensitivity and Precision

A standard curve was made (0.1-25μg/mL) in plasma with coefficient of determination 0.99 or greater. Using the criterion of signal to noise ratio of 2 the sensitivity for metronidazole was 0.01μg/mL. Within day coefficient of variation based on six determinations at 0.5,1 and 5μg/mL was less than 5%.

Bioavailability Study

The mean plasma concentration of metronidazole in ten volunteers after the oral ingestion of a 400mg tablet of metronidazole from two different sources is shown in Figure 7. Comparison of the metronidazole bioavailability parameters for the two different

dosage forms, using paired t-test showed no significant differences ($p>0.05$). The peak plasma level,time to peak plasma level and area under the curve were in the region of 8.4μg/mL, 1.1 hrs, and 100μg.hr/mL respectively for both dosage forms.

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