SUSCEPTIBILITY OF *TRICHOMONAS VAGINALIS* TO METRONIDAZOLE AND OTHER COMPOUNDS

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DECLARATION

This study represents original work by the author and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

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PLAGIARISM DECLARATION

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CONFERENCE PRESENTATIONS

Development of a molecular typing method for *Trichomonas vaginalis* – Oral presentation at the Lesedi Afrika '99 IUSTI/STD/HIV 6th World Congress and 38th IUSTI General Assembly (21-24 November 1999)

In vitro susceptibility testing of *Trichomonas vaginalis* to metronidazole – Oral presentation at the International Congress of Sexually Transmitted Diseases IUSTI/ISSTDR conference held in Berlin (24-27 June 2001)

Clinical response of *Trichomonas vaginalis* infection to metronidazole and *in vitro* susceptibility – Oral presentation at the Reproductive Health: Taking care of Tomorrow's World, Brisbane, Australia (2-5 July 2002)

Clinical response of *Trichomonas vaginalis* infection to metronidazole and *in vitro* susceptibility - Poster presentation at the South African AIDS Conference (3-6 August 2003)

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ABSTRACT

Trichomonas vaginalis is the most common sexually transmitted infection caused by a single known organism worldwide; and has been associated with an increased risk of HIV acquisition and transmission. Despite its high prevalence in South Africa, limited information is available on the extent of *T. vaginalis* metronidazole resistance and genotypic variation in this setting. We therefore tested the susceptibility of local *T. vaginalis* isolates against metronidazole and drugs prescribed in combination in the context of syndromic management of vaginal discharge syndrome. Susceptibility testing of 40 isolates demonstrated that metronidazole as well as some of the other drugs tested showed inhibiting effect on *T. vaginalis*. We recommend that these drugs be tested for synergistic effect with metronidazole. In a different set of 160 isolates the minimum inhibitory concentrations (MIC) of metronidazole ranged from 1.1 µg/ml to > 34.2 µg/ml (6.25 µM to > 200 µM) in the aerobic assay. Interpretation of these MICs differed based on the different resistance breakpoints applied. There was no correlation between MIC and treatment outcome in the subset of 56 patients that returned for follow-up. The expected association between MIC and clinical outcome was only observed in one of eight patients with unsatisfactory treatment outcome. This patient‘s isolate had the highest MIC. In the remaining seven patients with unsatisfactory treatment outcome, no relation with the susceptibility test result was found. A possible reason for the poor correlation may be inadequate concentration of metronidazole at the site of infection. In view of this, we assessed a self-administered and collected vaginal tampon specimen for the investigation of metronidazole concentration in the vagina of five healthy volunteers, using high performance liquid chromatography (HPLC). Maximum values of metronidazole concentrations detected in both serum and vaginal fluid were obtained at two hours

following oral administration of 2 g of the drug. This method can be applied in future clinical studies to correlate treatment outcome and MICs with metronidazole concentration at the site of infection. This may lead to the development of susceptibility assays and interpretation criteria that are better able to predict treatment outcome than the current methods. Another reason for the poor correlation between treatment outcome and *in vitro* resistance may be early reinfection. We used PCR-RFLP, targeting a 650-bp repeat region in the *T. vaginalis* genome, to genotype *T. vaginalis* isolates. Four genotypes were found in 100 *T. vaginalis* isolates using this method. Both the vaginal secretion of metronidazole and the strain typing methodology needs to be further investigated before a comprehensive study as outlined above can be executed.

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ABBREVIATIONS

°C	degree Celsius
µl	microliter
µM	micromolar
µm	micro meter
ATCC	American Type Culture Collection
bp	base pairs
BV	bacterial vaginosis
CDC	Centers for Disease Control and Prevention
C_{max}	maximum concentration
DMF	N, N-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
e.g.	example
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
g	gram
GLC	gas liquid chromatography
h	hour
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
M	molar
mg	milligram
MIC	minimum inhibitory concentration
min	minute
MLC	minimum lethal concentration
MLEE	multilocus enzyme electrophoresis
ml	millilitre
MLST	multilocus sequence typing
NAATs	nucleic acid amplification tests
NICD	National Institute for Communicable Diseases

nm	nanometre
L	litre
ng	nanogram
PCR	restriction fragment length polymorphism
PFGE	pulsed field gel electrophoresis
p.o	per oral
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
s	seconds
SAIMR	South African Institute for Medical Research
STI	Sexually transmitted infection
SEM	standard error of the mean
SNP	single nucleotide polymorphism
STDev	Standard deviation
TE	Tris-EDTA
TBE	Tris/Borate/EDTA
TMA	transcription-mediated amplification
TYM	trypticase–yeast–maltose
UV/VIS	ultraviolet/visible
United Kingdom	UK
United States of America	USA
V	volt
vs	versus
WHO	World Health Organisation

CHAPTER 1

1.1 INTRODUCTION

Trichomonas vaginalis is the causative agent of trichomoniasis, which is the most common, treatable non-viral sexually transmitted infection (STI) (Muzny and Schwebke, 2013). According to the World Health Organisation (WHO) an estimated 276.4 million new cases of trichomoniasis occurred globally in 2008 (WHO, 2012). Trichomoniasis prevalence rates in adults ranging from approximately 17% to 42% have been reported in eastern and southern Africa (Sturm *et al*., 1998; Watson-Jones *et al*., 2000; Buvé *et al*., 2001; Apalata *et al*., 2014). Various studies conducted in in South Africa reported very high *T. vaginalis* prevalence of approximately 15% to 42% (Sturm *et al*., 1998; Moodley *et al*., 2002a; Mlisana *et al*., 2012; Lewis *et al*., 2013; Apalata *et al*., 2014). *T. vaginalis* infection has been associated with an increased risk of transmission of other STIs, including human immunodeficiency virus (HIV) (Buvé *et al*., 2001, McClelland *et al*., 2007; Kissinger and Adamski, 2013). Given the high prevalence of *T. vaginalis*, this association could be responsible for a substantial number of HIV infections globally (Sorvillo and Kerndt, 1998).

Clinical manifestations of trichomoniasis in women range from asymptomatic carriage to profuse vaginal discharge, vulvar pruritis and dysuria. In men trichomonas infection manifests as urethritis with dysuria and mild pruritis but is mainly asymptomatic (Thomason and Gelbart, 1989; Hammil, 1989; Paterson *et al*., 1998; Cudmore *et al*., 2004). Some studies in men with urethritis report no association with the presence of *T. vaginalis* suggesting that symptomatology may be of short duration and self-limiting (Sturm *et al*, 2004). Pregnant

women infected with *T. vaginalis* are predisposed to premature labour, premature rupture of membranes and low-birth-weight infants (Petrin *et al*., 1998).

T. vaginalis can be detected in vaginal, prostatic and urethral secretions as well as in semen and urine. For long culture was considered the most reliable diagnostic method with a reported sensitivity of >90% for detecting *T. vaginalis* in some studies (Philip *et al*., 1987; Beverly *et al*., 1999), it requires complex media and is time-consuming. Various nucleic acid-based methods have since been developed to improve the specificity but in particular the sensitivity of the laboratory diagnosis (Shaio *et al*., 1997; Paterson *et al*., 1998; Nye *et al*., 2009; Andrea *et al*., 2011; Schwebke *et al*., 2011). However, culture is still essential to allow for susceptibility testing.

The only drugs proven to be effective against trichomoniasis are the 5-nitroimidazoles, specifically metronidazole and tinidazole (Secor, 2012). Metronidazole is for many years the principle drug used to treat trichomoniasis (Lofmark *et al*., 2010). The standard treatment is given orally in a single 2 g dose (Centers for Disease Control and Prevention [CDC], 2010). Most isolates of *T. vaginalis* are highly susceptible to metronidazole but resistance has been reported (Thurner and Meingassner, 1978; Kulda *et al*., 1982; Muller *et al*., 1988; Dunne *et al*., 2003; Schwebke and Barrientes, 2006; Upcroft *et al*., 2009; Bosserman *et al*., 2011; Kirkcaldy *et al.,* 2012; Rukasha *et al*., 2013). Several susceptibility testing methods for metronidazole have been described (Thurner and Meingassner, 1978; Ralph *et al*., 1983; Upcroft and Upcroft, 2001a), however the results obtained may not always correlate with response to treatment (Muller *et al*., 1988; Schwebke and Barrientes, 2006; Bosserman e*t al*., 2011). Consequently, how laboratory resistance translates in clinical resistance is still an unanswered question.

Treatment failure in patients infected with *T. vaginalis* may be due to early reinfection, non-adherence to treatment, poor absorption of the drug, failure of the drug to reach the site of infection or infection with a drug resistant strain (Kane *et al*., 1961; Petrin *et al*., 1998). Since trichomoniasis is a localised infection and metronidazole is administered systemically, the reasons for treatment failure may be better understood if the concentration of the drug at the site of infection is known and if reinfection between initiation of treatment and follow-up investigations could be excluded.

Several studies have described the use of high performance liquid chromatography (HPLC) to determine the metronidazole concentration in biological fluids (Galmier *et al*., 1998; Mustapha *et al*., 2006; Emami *et al*., 2006). Many of these studies reported on metronidazole levels in plasma, serum or urine. Only a few studies investigated metronidazole levels in vaginal secretions (Manthei *et al*., 1969 cited by Larsen *et al*., 1986; Davis *et al*., 1984; Larsen *et al*., 1986; Robertson *et al*., 1988).

To exclude reinfection, a reproducible and discriminatory typing method is required. Due to the worldwide high prevalence of trichomoniasis, there is a need to understand the genetic diversity of the organism that causes it (Cornelius *et al*., 2012). This has led to the development of an array of typing methods that aimed to characterize *T. vaginalis* isolates. Molecular typing methods for *T. vaginalis* previously reported include, but are not limited to, pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) analysis, random amplified polymorphic DNA (RAPD) analysis, and multilocus sequence (MLST) typing, (Upcroft *et al*., 2006a; Crucitti *et al*., 2008; Valadkhani *et al*., 2011; Cornelius *et al*., 2012; Meade and Carlton, 2013). Although these various methods have been used to characterize *T. vaginalis*, there is no "gold standard" method for typing *T. vaginalis* isolates (Cornelius *et al*., 2012).

Very few studies describing strain typing or antimicrobial susceptibility testing of South African isolates of *T. vaginalis* have been reported (Upcroft *et al*., 2006a; Upcroft *et al*., 2006b; Kock *et al*., 2013; Rukasha *et al*., 2013). Furthermore, the degree of metronidazole resistance in Kwazulu-Natal, the province of South Africa with the largest population, has not been previously investigated. In view of this the present study was designed, using isolates from selected cohorts of individuals from Kwazulu-Natal, to perform antimicrobial susceptibility testing; and to develop a molecular typing method to characterize *T. vaginalis* isolates. In addition, due to the limited information currently available on metronidazole concentrations in the vagina after standard oral therapy, we developed a method for determination of metronidazole levels in vaginal fluid.

1.2 AIMS OF THIS STUDY

- To perform antimicrobial susceptibility testing using the broth macro-dilution method against syndromic management drugs; and to determine the susceptibility of *T. vaginalis* isolates to metronidazole under both aerobic and anaerobic conditions using the broth micro-dilution method.
- To develop a method for determination of metronidazole concentration in vaginal secretions using HPLC.
- To use PCR-RFLP genotyping to characterize *T. vaginalis* isolates

Accordingly, this thesis has been divided into three components: antimicrobial susceptibility testing of *T. vaginalis*, the development of a methodology using HPLC to detect metronidazole concentration in vaginal secretions and PCR-RFLP genotyping to characterize *T. vaginalis* isolates.

CHAPTER 2

REVIEW OF LITERATURE

2.1 *Trichomonas vaginalis*

2.1.1 History

Trichomonas vaginalis is the cause trichomoniasis which is one of the most common sexually transmitted infections (Bachmann *et al*., 2011; Hillier, 2013). This protozoan was first observed and described in 1836 by a French physician, Alfred Donné, who considered it an etiologic agent of vaginitis. In 1896, Dock cited by Thomason and Gelbart, 1989, questioned whether the protozoan was a pathogen, since males and females harbouring the organism were frequently asymptomatic. It was finally demonstrated in 1916 by Hoehne, that by eradicating the parasite in symptomatic patients, one would eliminate the resultant vaginitis. The organism was again ignored until Johnson *et al*., 1943 cited by Thomason and Gelbart, 1989, developed a culture medium that allowed study of the organism in an axenic environment, free of bacteria and fungi. This cysteine-peptone-liver extract-maltose medium later evolved into Kupferberg's medium, developed in 1948 (Gelbart *et al*., 1990). Diamond's trypticase-yeast-maltose (TYM) medium was developed in 1957 (Diamond, 1957 cited by Gelbart *et al*., 1990). The development of a culture medium made it possible to test the protozoan for antibiotic susceptibility. Thus in 1959, Cosar and Julou cited by Thomason and Gelbart, 1989, discovered that metronidazole kills the organism.

2.1.2 Morphology and structure

T. vaginalis is a unicellular, flagellated protozoan which resides extracellularly in the lower

genitourinary tract of human beings, it's only natural host (Schwebke and Burgess, 2004). *T. vaginalis* cells vary in size and shape (Petrin *et al*., 1998). The average size is approximately 10 by 7 μm and it generally appears pyriform or ovoid (Petrin *et al*., 1998). The shape of the organism is more uniform in axenic culture (Figure 2.1[A]), but it appears in an amoeboid form when attached to vaginal epithelial cells (Figure 2.1 [B and C]) (Petrin *et al*., 1998).

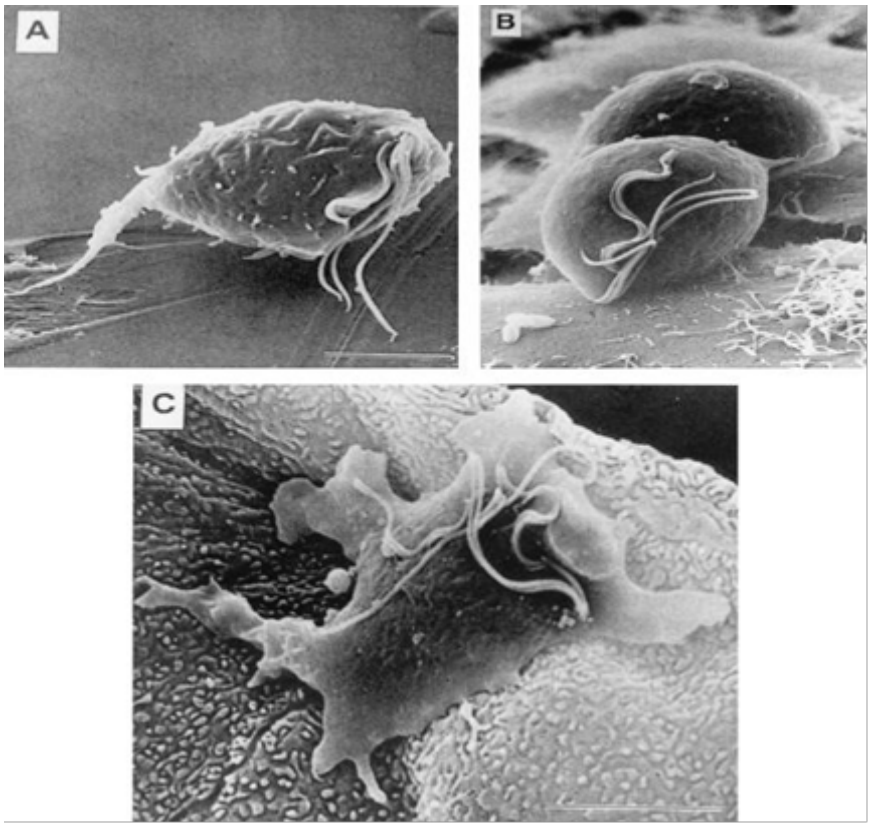


Figure 2.1: (A) *T. vaginalis* in broth culture with visible axostyle, undulating membrane and flagella. (B) *T. vaginalis* on the vaginal epithelial cell surface before amoeboid transformation (C) Amoeboid form of *T. vaginalis* in cell culture using vaginal epithelial cells (Arroyo *et al*., 1993 cited by Petrin *et al*., 1998)

The organism has five flagella. Four flagella are situated at its anterior pole while the fifth is, incorporated within the undulating membrane which extends along the body of the parasite (Petrin *et al*., 1998) (Figure 2.1[A]). The flagella and undulating membrane give this parasite a

characteristic jerky movement as well as rapid motility (Petrin *et al*., 1998). *T. vaginalis* can gather and internalize the flagella under unfavourable growth conditions (Petrin *et al*., 1998). The cytoplasmic costa is located beneath the undulating membrane. The axostyle, a rigid structure runs its entire length, protrudes from the posterior pole and bisects the organism longitudinally (Petrin *et al*., 1998; Schwebke and Burgess, 2004). Similar to eukaryotic cells, the nucleus which is located towards the anterior portion of the organism is enclosed by a porous nuclear envelope (Petrin *et al*., 1998). A Golgi apparatus resembling a parabasal body is situated in the cytoplasm adjacent to the nucleus. Multiple granular organelles called hydrogenosomes (Benchimol *et al*., 1996), which are unique to trichomonads, are found along the axostyle and costa. Hydrogenosomes are involved in the organisms's metabolic pathways (Dunne *et al*., 2003). These organelles generate hydrogen by converting pyruvate to acetate, carbon dioxide, and hydrogen. The reduced hydrogenosomal protein, ferredoxin, may be involved in the maintenance of an anaerobic environment within the intracellular space (Cudmore *et al*., 2004).

Whether *T. vaginalis*, like most pathogenic protozoa, has a life cycle has not been resolved (Sood and Kapil, 2008). It is generally accepted that the organism exists only in the trophozoite form and is unable to form cysts (Petrin *et al*., 1998). The absence of a phase that is environmentally resistant is thought to explain its mode of transmission by close bodily contact. Some reports have however suggested that the organism may assume a pseudocyst form under unfavourable environmental conditions (Pereira-Neves *et al*., 2003). The organism reproduces by longitudinal binary fission (Petrin *et al*., 1998).

2.1.3 Metabolism and Growth

T. vaginalis have hydrogenosomes which play a similar role as mitochondria in aerobically metabolising eukaryotic cells (Lindmark and Muller, 1973 cited by Bradley *et al*., 1997). These organelles produce hydrogen, process carbohydrates (Petrin *et al*., 1998), metabolize pyruvate and produce adenosine triphosphate (ATP) through substrate-level phosphorylation (Bradley *et al*., 1997). Three proteins: pyruvate:ferredoxin oxidoreductase, ferredoxin and iron-only [Fe]-hydrogenase, are sequentially involved in the metabolism of pyruvate (Gehrig and Efferth, 2009). *T. vaginalis* does not have metabolic pathways required for the synthesis of sterols and fatty acids (Beach *et al*., 1990). Therefore, the organism has mechanisms to facilitate the uptake of plasma proteins and lipoproteins necessary for its growth (Peterson and Alderete, 1982; Peterson and Alderete, 1984).

T. vaginalis is unable to synthesize many macromolecules, such as purines, pyrimidines and many lipids. It therefore needs another organism that is able to provide these molecules and is thus an obligate parasite (Petrin *et al*., 1998). *T. vaginalis* obtains these substances from vaginal secretions or via phagocytosis of bacteria, yeasts, and erythrocytes (Heine and McGregor, 1993). Culture media for *T. vaginalis* should therefore include all essential macromolecules, vitamins and minerals (Petrin *et al*., 1998). The most widely used culture medium for *T. vaginalis* is Diamonds TYM medium (Clark and Diamond, 2002) which contains horse serum as a main source of organic substances. Optimal growth of *T. vaginalis* occurs under anaerobic conditions, at a pH of 6.0 to 6.3 (Diamond, 1986 cited by Petrin *et al*., 1998).

2.1.4 *Trichomonas vaginalis* Virus (TVV)

The *Trichomonas vaginalis* double stranded RNA virus (TVV) was first identified by Wang and Wang in 1985. (Wang and Wang, 1985a). The size of the viral RNA strand has been reported to vary between 4.3 and 5.5 kb (Wang and Wang, 1985b; Khoshnan and Alderete, 1993). *T. vaginalis* isolates can be infected with more than one strain of TVV concurrently (Benchimol *et al*., 2002). Goodman and colleagues found two *T. vaginalis* isolates to be concurrently infected by four strains of TVV (Goodman *et al*., 2011). TVV was shown to be present in *T. vaginalis* isolates obtained from various different countries including South Africa (Snipes *et al*., 2000; Weber *et al*., 2003; Fraga *et al*., 2005; Kim *et al*., 2007; Goodman *et al*., 2011; Heidary *et al*., 2013). TVV was detected in 59 of the 72 (81.9%) isolates collected from hospitals in Ga-Rankuwa and Cape Town, SA (Weber *et al*., 2003). Although the biological functions of the TVV genetic information is not known (Goodman *et al*., 2011), it was previously suggested that this virus may be associated with the expression of P270, a highly immunogenic *T. vaginalis* surface protein (Khoshnan and Alderete 1994; Kim *et al*., 2007) and with the expression of cysteine proteinases (Provenzano *et al*., 1997). *T. vaginalis* isolates have been divided into two types based on the presence or absence of the TVV: isolates not infected with TVV are designated Type I and those infected with TVV are designated Type II (Wendel *et al*., 2002)

2.2 EPIDEMIOLOGY

The total number of new cases of *T. vaginalis* in 2008 in adults between the ages of 15 and 49 was estimated to be 276.4 million (WHO, 2012). There is currently however very limited population based data to inform these global estimates (Poole and McClelland, 2013). *T.*

vaginalis has a worldwide distribution and has been found in all age, racial and socioeconomic groups (Sood and Kapil, 2008; Leon *et al*., 2009; Madhivanan *et al*., 2009; Napierala *et al*., 2010; Apalata *et al*., 2014). Both men and women may be infected, although the prevalence is higher in women (Eshete *et al*., 2013). However, this may be an underestimation since most infections in male are asymptomatic suggesting the presence of low numbers of organisms that easily escape detection (Hobbs *et al*., 2006). The prevalence of *T. vaginalis* has been shown to increase with age in males and females (Joyner *et al*., 2000; Ginocchio *et al*., 2012). Older individuals may not consider themselves at risk and may therefore engage more frequently in unprotected sex (Olivi *et al*., 2008), which could then lead to acquisition of STIs. Another explanation may be that repeated infection leads to milder symptomatology and consequently delayed or no treatment.

Trichomoniasis has been associated with a 1.5 (1.5–3.0) times increased risk of HIV acquisition (Lazenby, 2011). Several recent studies have reported this epidemiologic synergy between *T. vaginalis* and HIV (McClelland *et al*., 2007; Shafir *et al*., 2009; Napierala *et al*., 2010). The actual burden of the disease in South Africa, where HIV is also endemic, is unknown. Available prevalence data are from studies carried out in selected populations that may not be representative of the total population. In a study by Moodley *et al*., 2003, prevalence of *T. vaginalis* in South African women attending a reproductive health clinic was 29%. More recently in South Africa, there were reports of a prevalence of 6.1% in men and 23.6% in women attending a primary health care clinic in Johannesburg (Lewis *et al*., 2013). In Durban a prevalence of 20.3% was found amongst a cohort of high-risk HIV-negative female sex workers (Mlisana *et al*., 2012), and in approximately 16% of 198 female patients attending a primary health care facility with vaginal discharge syndrome (Apalata *et al*., 2014).

2.3 TRANSMISSION

The pathogen is transmitted during sexual intercourse. The organism inhabits the genitourinary tract and thrives on the surface of squamous epithelial cells lining the vagina, urethra and Skene glands in women and in the seminal vesicles, urethra and prostate gland in men. It can also be found in the preputial sac of uncircumcised males (Thomason and Gelbart, 1989). The incubation period in women, has been reported to vary from 4 to 28 days in about 50% of infected individuals. When assessed within 48 hours after sexual contact with an infected female, it was shown that 70% of males carried the organism (Thomason and Gelbart, 1989). Non-sexual transmission of *T. vaginalis* is theoretically possible. One study reported a high prevalence of *T. vaginalis* in virgins and the authors suggested that the organism could have been transmitted through shared bathing water and poor hygiene (Crucitti *et al*., 2011). Given that the organism is so site-specific, non-sexual transmission is believed to be a rare occurrence (Thomason and Gelbart, 1989).

2.4 CLINICAL MANIFESTATIONS

T. vaginalis colonises the squamous epithelium of the female genital tract, however it can be recovered from the urethra, and has been found in the internal genitalia of female as well (Petrin *et al*., 1998; Moodley *et al*., 2002b). Symptomatic women may present with signs of profuse vaginal discharge, malodour, labial edema, erythema and/or vulvar pruritis and with symptoms of dysuria, and/or dyspareunia (Petrin *et al*., 1998). More than half of the symptomatic women present with vaginal discharge. The type of discharge depends on the presence of associated bacteria. There is a strong association with trichomoniasis and bacterial vaginosis because *T. vaginalis* creates an anaerobic environment and an increase in vaginal pH, thereby changing the

vaginal flora (Thomason *et al*., 1988). Less than 10% of symptomatic women present with classic green, frothy, foul-smelling discharge and, punctate haemorrhagic lesions on the cervix (strawberry cervix) are detected in approximately 2% of infected women (Petrin *et al.*, 1998). It has been reported that symptoms often are first noticed or are exacerbated at menstruation or after sexual intercourse (Grist *et al*., 1987). Adverse pregnancy outcomes such as premature rupture of membranes and preterm birth have been associated with *T. vaginalis* infection (Cotch *et al*., 1997). It has been shown in previous studies that 25-50% of women with *T. vaginalis* are asymptomatic (Schwebke and Burgess, 2004; Seña et *al*., 2007; Piperaki *et al*., 2010)

Trichmoniasis is asymptomatic in 40% to 75% of men (Schwebke and Hook, 2003; Seña *et al*., 2007) and its role in male urethritis syndrome remains obscure (Sturm *et al*., 2004). Symptomatic men usually complain of urethral discharge, dysuria, mild pruritis, or burning directly after sexual intercourse (Thomason and Gelbart, 1989). The lower numbers of organisms found in males has been associated with the presence of zinc in prostatic fluid (Kriege and Rein, 1982). Zinc has antitrichomonal activity *in vitro* and it has therefore been postulated that it inhibits proliferation of *T. vaginalis in vivo*.

2.5 DIAGNOSIS

Several methods including microscopy, culture, antigen detection tests and nucleic acid amplification tests have been used for diagnosis of trichomoniasis (Hobbs and Seña, 2013). The organism can be detected in vaginal, urethral and prostatic secretions as well as in semen and urine. A commonly applied diagnostic method for diagnosis of *T vaginalis* infection in women is microscopic examination of a wet mount preparation of vaginal secretions mixed with normal saline (Hobbs and Seña, 2013). Observation of the pear-shaped trichomonads with their

distinctive motility is considered highly specific. However when compared to nucleic acid amplification tests, the sensitivity of microscopy ranges from 44% to 68% (Huppert *et al*., 2007; Patil *et al*., 2012). Microscopic examination of male urethral specimens or urine sediment has low sensitivity for detection of *T vaginalis* infection in men due to the presence of fewer organisms (Hobbs *et al*., 2006).

Culture is more sensitive than direct microscopic examination (Hobbs and Seña, 2013). Cultures of vaginal specimens from women with trichomoniasis are usually positive after three days of inoculation but it can take up till seven days before growth is detected. Time-to-positivity depends on the concentration of organisms in the specimen. Therefore, delayed diagnosis by culture is seen particularly in asymptomatic women and in men (Garber, 2005). Cultures must be microscopically examined daily for five to seven days before being regarded as negative (Hobbs *et al*., 2006). To simplify diagnosis by culture, the InPouch system (BioMed Diagnostics, USA) was developed (Borchardt and Smith, 1991). The system consists of a flexible oxygen impenetrable plastic pouch which contains the culture medium. The contents can be microscopically examined daily without opening the pouch (Borchardt and Smith, 1991).

More recently rapid, non-culture diagnostic tests that detect *T vaginalis* antigens or nucleic acids were developed (Hobbs and Seña, 2013). The commercially available antigen detection tests include the OSOM *Trichomonas* Rapid Test (Sekisui Diagnostics, California, USA) and the TV latex agglutination test (Kalon Biological, Surrey, UK). Affirm VPIII (Becton Dickinson, Maryland, USA) detects nucleic acid by probe hybridisation without amplification. This test detects *T vaginalis*, *Gardnerella vaginalis* and *Candida albicans* simultaneously (Andrea and

Chapin, 2011).

As for many infectious diseases, nucleic acid amplification tests (NAATs) now provide highly sensitive tools for diagnosis of *T. vaginalis* infection. NAATs find their origin in polymerase chain reaction (PCR) technology and include classic PCR, transcription-mediated amplification (TMA) and other technical variations that amplify specific trichomonal DNA or RNA target sequences (Hobbs and Seña, 2013). Because these methods increase the copy numbers of the diagnostic marker, the analytical sensitivity of NAATs is higher than that of microscopy, culture, antigen detection or nucleic acid probe assays (Hobbs and Seña, 2013), which all depend on the concentration of organisms in the specimens. The TMA-based APTIMA® *T. vaginalis* assay (Hologic Gen-Probe Inc, California, USA) received the EU CE mark and US Food and Drug Administration (FDA) clearance for *in vitro* detection of *T. vaginalis* in specimens from women (Schwebke *et al*., 2011). *T. vaginalis* NAAT sensitivities range from 76% to 100% (Nye *et al*., 2009, Andrea and Chapin, 2011, Schwebke *et al*., 2011). Although NAATs may eventually replace less sensitive tests for diagnosis of *T vaginalis* infection, culture will continue to be important in cases of persistent infection or suspected treatment failure for which antimicrobial susceptibility testing of clinical isolates may be necessary (Hobbs and Seña, 2013).

2.6 TREATMENT

The only drugs proven to be effective for treatment of *T. vaginalis* are the 5-nitroimidazoles of which metronidazole and tinidazole have proven efficacy in clinical trials (Robertson *et al*., 1988; Forna and Gülmezoglu, 2007). The single dose metronidazole regimen was first used in 1971 (Csonka, 1971 cited by Gabriel *et al*., 1981). Subsequently, other studies were conducted

to demonstrate efficacy of the 2 g oral dose of metronidazole (Thin *et al*., 1979; Gabriel *et al*., 1981). This treatment regimen with a 2 g single dose of metronidazole is usually uncomplicated and is well tolerated (Samuelson, 1999). A single 2 g oral dose of either metronidazole or tinidazole is currently used for treatment of trichomoniasis in both men and women (Muzny and Schwebke, 2013). It is recommended that both partners are treated (Centers for Disease Control [CDC], 2010), even if one of them is asymptomatic. Despite reports on teratogenicity in animals (Chacko and Bhide, 1986), single dose oral metronidazole has been proven to be safe in pregnancy, including the first trimester (Burtin *et al*., 1995).

According to the CDC, individuals can be treated with 500 mg of metronidazole twice daily for seven days if treatment failure occurs with a 2 g dose of metronidazole, and reinfection has been excluded (CDC, 2010). Treatment with a 2 g oral dose of metronidazole or tinidazole once daily for five days should be considered in the event that this regimen fails (CDC, 2010).

A 7-day regimen of metronidazole 500 mg orally twice daily may be preferred for the treatment of *T vaginalis* in HIV-infected women (Muzny and Schwebke, 2013). A recent study showed that single-dose therapy with metronidazole was not as effective as the 7-day regimen in a cohort of HIV positive women (Kissinger *et al*., 2010). On the contrary a study conducted among women attending a primary healthcare clinic in SA showed that microbiologic cure rates for *T. vaginalis* were independent of HIV status after 8 to 10 days following treatment with a 2 g stat dose of metronidazole (Moodley *et al*., 2003). A similar finding was reported in a recent prospective cohort study (Balkus *et al*., 2013).

Treatment options are still limited in patients with treatment resistance and/or nitroimidazole

allergy. The use of numerous alternative chemotherapeutic agents has been reported (Cedillo-Rivera *et al*., 2002, Camuzat-Dedenis *et al*., 2001; Bouma *et al*., 1998), however there is currently no proven effective alternative ((Munzy and Schwebke, 2013).

2.7 RESISTANCE TO 5-NITROIMIDAZOLES

Although metronidazole is the drug of choice for treatment of trichomoniasis, resistant *T. vaginalis* isolates have been isolated (Thurner and Meingassner, 1978; Muller *et al*., 1980; Dunne *et al*., 2003). The reported prevalence of metronidazole resistance varies from 2 to 17% in women (Schmid *et al*., 2001; Schwebke and Barrientes, 2006; Kissinger *et al*., 2008; Upcroft *et al*., 2009; Krashin *et al*., 2010; Kirkaldy *et al*., 2012; Rukasha *et al.*, 2013). *In vitro* drug resistance amongst *T. vaginalis* is ubiquitous (Meri *et al*., 2000; Perez *et al*., 2001; Lo *et al*., 2002; Dunne *et al*. 2003; Upcroft *et al*., 2009; Bosserman et al., 2011; Kirkaldy et al., 2012; Rukasha *et al*., 2013) however accurate data on the magnitude of clinically resistant trichomoniasis are not available (Sood and Kapil, 2008). It is also important to note that it is difficult to differentiate between reinfection and resistance (Van Der Pol *et al*., 2005). So far, understanding of the exact mechanism of resistance in *T. vaginalis* isolates to the 5-nitroimidazoles is limited (Secor, 2012).

Organisms isolated from patients with treatment failure usually display increased minimal inhibitory concentrations (MIC) of metronidazole in susceptibility assays conducted aerobically (Kulda *et al*., 1982; Lossick *et al*., 1986; Muller *et al*., 1988; Rasoloson *et al*., 2002; Dunne *et al*., 2003).

2.7.1 *In vitro* metronidazole resistance

Metronidazole penetrates *T. vaginalis* by means of passive diffusion and results in the release of cytotoxic nitro anion radicals via anaerobic reduction (Sood and Kapil, 2008). Drug activation occurs in the hydrogenosome where the drug is reduced by pyruvate: ferredoxin oxidoreductase (PFOR). Since the concentration difference of the non-active unprocessed molecule drives the diffusion process, the activation of the drug facilitates the entry of more drug into the organism and the accumulation of cytotoxic radicals (Land and Johnson, 1999; Sood and Kapil, 2008). Leitsch and colleagues described an alternate pathway for 5-nitroimidazole drug activation (Leitsch *et al*., 2009). They suggested that the nitroreductase activity of the flavin dependent enzyme thioredoxin reductase activates the 5-nitroimidazole drugs, including metronidazole, to their toxic form in the *T. vaginalis* cytoplasm (Leitsch *et al*., 2009).

Metronidazole MICs obtained under aerobic and anaerobic incubation conditions differ (Kulda, 1999; Rasoloson *et al*., 2002). MICs have been shown to be higher in susceptibility assays carried out aerobically compared to those carried out anaerobically (Upcroft and Upcroft, 2001a; Upcroft and Upcroft, 2001b). This is due to various mechanisms of drug action (Dunne *et al*., 2003; Gehrig and Efferth, 2009).

Aerobic resistance

This type of resistance is characterised by impaired oxygen scavenging processes (Rasoloson *et al*., 2001) and represents the earliest stage in resistance (Dunne *et al*., 2003; Gehrig and Efferth, 2009). This process results in an increased oxygen concentration inside the organism impairing the low redox potential necessary for metronidazole activation (Gehrig and Efferth, 2009).

Anaerobic Resistance

It has been reported that clinical isolates also exhibit resistance under anaerobic conditions (Upcroft and Upcroft, 2001b; Dunne *et al*., 2003; Voolmann and Boreham, 1993 cited by Cudmore *et al*., 2004). Resistance under anaerobic conditions can be developed *in vitro* by continuously exposing the organisms to increasing concentrations of metronidazole (Dunne e*t al*., 2003). This type of resistance is independent of oxygen scavenging (Gehrig and Efferth, 2009) and is associated with decreased expression levels of the hydrogenosomal enzymes involved in drug activation, i.e. PFOR, malic enzyme, NADH:ferredoxin oxidoreductase (NADH:FOR), ferrodoxin and FE-hydrogenase (Rasoloson *et al*., 2002; Gehrig and Efferth, 2009). Ferredoxin gene mutations were also detected in clinical isolates of *T. vaginalis* (Heidari *et al*., 2013). It was however concluded that additional *in vitro* and *in vivo* studies are required to determine the association between ferredoxin gene mutation and resistance to metronidazole in clinical isolates of *T.vaginalis*.

2.8 ANTIBIOTIC SUSCEPTIBILITY TESTING

Antimicrobial susceptibility testing is done in order to assess susceptibility to antimicrobial agents, and to detect possible drug resistance in organisms (Jorgensen and Ferraro, 2009). It provides information to prescribers on the choice of appropriate antimicrobial drugs for therapy or prophylaxis of infections in specific patients, or to aid in antimicrobial policy formulation (MacGowan and Wise, 2001). Data generated from susceptibility testing may also be used to track the occurrence and prevalence of antimicrobial resistance within specific geographical areas (Macgowan and Wise, 2001). Commonly used susceptibility testing methods include broth dilution tests, agar dilution tests, antimicrobial gradient diffusion, disk diffusion tests, and automated instrument systems (Jorgensen and Ferraro, 2009).

The broth dilution tests are used as susceptibility testing methods for *T. vaginalis*. The broth macro-dilution method is one of the earliest antimicrobial susceptibility testing methods that were developed (Ericsson and Sherris, 1971). This involves preparing two-fold dilutions of antibiotics (e.g. 16, 8, 4, 2, and 1 µg/ml) in a liquid growth medium dispensed into test tubes (Cates, 1999). The tubes containing the antibiotic are inoculated with a standardised suspension of organism depending on the type organism tested. Following incubation at the prescribed time and temperature the tubes are examined for visible growth of the organism (Andrews, 2001). The accuracy of this method is considered to be ± one two-fold concentration due mainly to the practice of manually preparing serial dilutions of the antibiotics (Balows, 1972 cited by Jorgensen and Ferraro, 2009).

Broth dilution testing became more popular and practical after the method was miniaturized by use of small, disposable, plastic "micro-dilution" trays (Jorgensen and Ferraro, 2009). A standard 96-well tray contains a volume of 200 µl per well and allows approximately 8 antibiotics to be tested in a range of 10 two-fold dilutions (two wells in each row are used for controls) (Jorgensen and Turnidge, 2007). Micro-dilution allows automation. Antimicrobial test panels are prepared using dispensing instruments, e.g. multi-channel pipettes that aliquot exact volumes of broth containing pre-weighed and diluted antibiotics into the individual wells of the tray. The panels are similarly inoculated with the standardised suspension of organisms. Many trays can be prepared from a single master set of dilutions (Jorgensen and Ferraro, 2009). Following incubation each of the wells is inspected for growth using a manual or automated viewing device.

2.8.1. Breakpoints and interpretation of *in vitro* susceptibility tests

Drug susceptibility of a microbe may be assessed by measuring the minimal inhibitory concentration (MIC) and/or minimum lethal concentration (MLC) of the drug for the organism being tested (Mayers, 2009). The MIC is the lowest concentration of an antimicrobial that prevents multiplication of the organism (Andrews, 2001). The MLC is the lowest concentration of drug at which all organisms are killed (i.e. no growth on subculture) (Mayers, 2009). MICs are considered the "gold standard" for determining the susceptibility of organisms to antimicrobials (Andrews, 2001). Diagnostic laboratories use MICs to confirm resistance and to provide a definitive answer when a borderline result is obtained by other testing methods (Andrews, 2001). The range of antibiotic concentrations used for determining MICs is universally accepted to be in doubling dilution steps up and down from 1 µg/ml.

In susceptibility testing a breakpoint is defined as "a discriminating concentration used in the interpretation of results of susceptibility testing to define isolates as susceptible, intermediate or resistant" (MacGowan and Wise, 2001). The rationale for determining a breakpoint is based on the assumption that an organism defined as "susceptible" should respond to the standard antibiotic dose, a "resistant" organism should not respond, and an "intermediate" one may or may not respond to standard doses, yet would have an increased chance to respond to a greater dose if the infection is at a site where the antimicrobial is actively concentrated (MacGowan and Wise, 2001). However this information regarding concentration of the drug at the site of infection is often not known (MacGowan and Wise, 2001). For most antimicrobial drugs two breakpoints are recognised. The breakpoint for resistance determines the value above which test organisms are labelled "resistant" while the breakpoint for susceptibility represents the value below which these are "susceptible". In between these two breakpoints is the

intermediate zone.

Ideally breakpoints should be determined by comparing response to treatment to a drug with the MIC values for that same drug. However, reports on such studies are rare (Moodley *et al*., 2002c). There are two main reasons for that. Firstly, such studies need to be done in a cohort that includes patients with infections caused by susceptible as well resistant organisms. However, treating patients with an antimicrobial drug to which the causative organism is resistant is unethical. The second problem is that such studies need to be done in patients with the same disease caused by the same organism while many infections can have different aetiology. Furthermore, since the patient's clinical condition at the initiation of treatment influences outcome, patients need to be stratified according to severity of disease. For these reasons, breakpoints used are established by consensus and are largely extrapolations based on blood concentrations achieved in volunteers combined with poorly controlled clinical observations.

As for most infections, it has also been reported that some patients with trichomoniasis infected with an *in vitro* "resistant" organism respond well to standard metronidazole therapy, and others with a "susceptible" organism fail to respond (Schwebke and Barrientes, 2006; Bosserman *et al*., 2011). There is no standardised *in vitro* assay (Mayers, 2009) or no agreed breakpoints available for susceptibility testing of *T. vaginalis*. Given the different types of susceptibility assays used, and mixed reporting of either MIC or MLC values, direct comparisons between studies is challenging (Dunne *et al*., 2003).

2.8.2 Antimicrobial susceptibility testing of *T. vaginalis*

Broth macro-dilution and broth micro-dilution are the methods commonly used for antimicrobial susceptibility testing of *T. vaginalis* (Upcroft and Upcroft, 2001a). Several studies reported on *in vitro* susceptibility to metronidazole (Muller *et al*., 1988; Meri *et al*., 2000; Snipes *et al*., 2000; Perez *et al*., 2000; Stiles *et al*., 2000; Upcroft and Upcroft, 2001a; Crowell *et al*., 2003; Schwebke and Barrientes, 2006; Kissinger *et al*., 2008; Upcroft *et al*., 2009; Bosserman *et al*., 2011; Kirkaldy *et al*., 2012; Rukasha *et al*., 2013). These studies used a variety of different MIC or MLC breakpoints to define "susceptible", "intermediate" and "resistant" isolates. Upcroft and Upcroft (2001a) developed a modified broth micro-dilution method, and recommended MIC breakpoints based on tests performed on *T. vaginalis* isolates from known responders and non-responders (See Chapter 4, Table 4.10). The authors suggest that this method with accompanying breakpoints may have the potential to be used widely for the determination of antimicrobial susceptibility in *T. vaginalis*. (Upcroft and Upcroft, 2001a). This needs to be verified by means of a prospective study that compares clinical outcome with the MIC of the organism isolated from the same patient.

Several studies have described the relationship between susceptibility of *T. vaginalis* to metronidazole and treatment outcome (Muller *et al*., 1988; Schwebke and Barrientes, 2006; Bosserman *et al*., 2011). An early study reported that *T. vaginalis* isolates exhibiting high MLCs determined by aerobic or anaerobic incubation were eradicated by standard metronidazole treatment (Muller *et al*., 1988). Bosserman and colleagues recently assessed the utility of susceptibility testing for patient care (Bosserman *et al*., 2011). Using a breakpoint for susceptibility of < 50 μg/ml, they reported that one-third of the isolates from women presenting with treatment failure were susceptible to metronidazole in the *in vitro* susceptibility assay.

The authors suggested that investigation of attainable intravaginal nitroimidazole concentrations in women with clinical treatment failure may contribute to a better understanding of this issue (Bosserman *et al*., 2011).

Given that there is a limited number of drugs available for the treatment of *T. vaginalis*, several studies measured the activities of various antimicrobial agents against metronidazole susceptible and metronidazole resistant isolates (Bouma *et al*., 1998; Upcroft *et al*., 1999; Malagoli *et al*., 2002; Crowell *et al*., 2004; Blaha *et al*., 2006; Calzada *et al*., 2007; Goodhew and Secor, 2013; Afonso Silva Rocha *et al*., 2014; Badawy *et al*., 2014). Although a few of these antimicrobial agents tested showed some activity (Chapter 4: Introduction), further investigations are still warranted.

2.9 DETECTION OF METRONIDAZOLE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

2.9.1 High Performance Liquid Chromatography

The purpose of quantitative analysis of body fluids for any drug is to establish the distribution of that drug in the body. This is usually done by monitoring drug concentrations during the dose-time interval. High performance liquid chromatography (HPLC) is most commonly used to measure drugs and their metabolites in body fluids such as blood and urine (Nikolin *et al*., 2004). HPLC has also been used for the determination of metronidazole concentrations in such fluids (Emami *et al*., 1999).

HPLC has been developed to quantitate chemical substances in fluids and is capable of identifying different chemicals in a sample containing a mix (Kupiec, 2004; Olbrich and Corbett, 2013). HPLC is a process by which outcome is determined by numerous variables such as column choice, temperature, mobile phase composition and pH (Bird, 1989; Olbrich and Corbett, 2013). These various components need to be optimised for the detection of each individual compound and sample type (Olbrich and Corbett, 2013). As shown in the schematic diagram in Figure 2.2 the HPLC system includes a pump, an injector, a column, a detector and a display system (Kupiec, 2004; Summerfield, 2010).

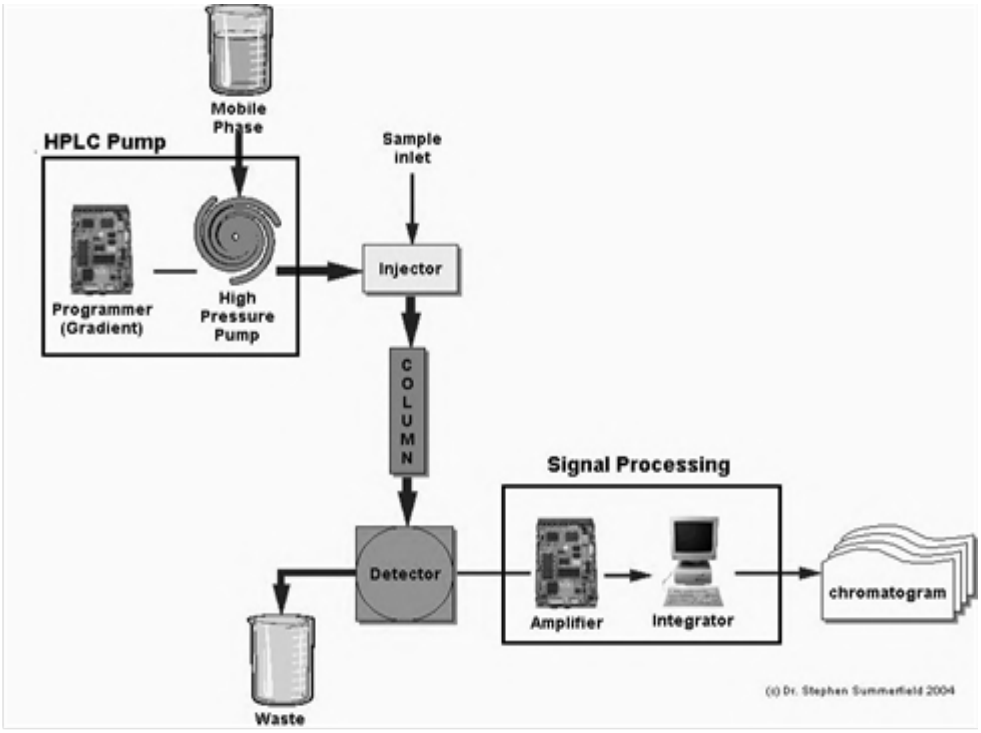


Figure 2.2: A schematic diagram of a HPLC system (Summerfield, 2010)

Pumps used in chromatography need to be able to generate high pressures of up to 6000 psi, to generate flow rates ranging from 0.1 to 10 ml/min with a flow reproducibility of 0.5 % or lower, to have pulse-free output and to be resistant to corrosion by the solvents used in the process (Skoog *et al*., 2004). HPLC involves the injection of the prepared sample into the

column which then retains the required compounds from the mobile phase (Olbrich and Corbett, 2013). Samples are injected into the column by means of a sampling loop or an autosampler containing an automatic injector (Skoog *et al*., 2004).

There are two ways of sample elution, i.e. isocratic and gradient elution. A single mobile phase is used for the entire analysis with the isocratic method. The gradient method involves changing the composition (two or more) of the mobile phase during the separation. The flow rate of the mobile phase must be optimized to ensure efficient separation of the compound from the background. Mobile phases usually comprise of water, acetonitrile, and/or methanol. (Olbrich and Corbett, 2013).

During HPLC separation takes place in the column which contains the stationary phase, which determines the efficacy of the HPLC method (Olbrich and Corbett, 2013). Columns are usually made of stainless steel tubing and are between 10-30 cm in length (Skoog *et al*., 2004) with an inside diameter of 3-5 mm (Summerfield, 2010). Columns are densely packed with silica particles leaving a pore size ranging from 3-10 µm (Skoog *et al*., 2004). Hydrocarbon chains of variable length are bonded to these silica particles. The most popular columns are the octadecyl carbon chain (C18) bonded silica and the C8-bonded silica. There are two types of columns used in HPLC systems: normal phase and reverse phase columns (Olbrich and Corbett, 2013). Reversed phase columns make use of hydrophobic columns and aqueous moderately polar mobile phases while normal phase columns are hydrophilic and use non-polar and non-aqueous mobile phases (Bird, 1989; Kupiec, 2004). Reverse phase columns are used more frequently than normal phase columns mainly because of the extensive range of substances that can dissolve in the mobile phase. The C8 and (C18) columns are commonly

used in reversed-phase HPLC (Olbrich and Corbett, 2013). A guard column is positioned in front of the analytical column. The guard column increases the lifespan of the analytical column by removing contaminants and particulate matter from the solvents (Skoog *et al*., 2004).

The retention or elution volume is the amount of mobile phase needed to draw the sample through the column (Kupiec, 2004). The retention time refers to the length of time a sample is held in the column by the stationary phase in relation to the period it resides in the mobile phase (Kupiec, 2004). The ability of the column to separate peaks on a chromatograph is referred to as the resolution (Kupiec, 2004).

The detector senses the presence of a compound passing through the column and measures its concentration. It provides a quantitative electronic signal to the data acquisition system (Kupiec, 2004). Detectors based on absorption of ultraviolet radiation are commonly used in liquid chromatography (Skoog *et al*., 2004). The current HPLC systems use a computerised data acquisition system. This system integrates the response of the detector and displays it as a chromatograph which may then be analysed (Kupiec, 2004).

2.9.2 Detection of metronidazole by HPLC

Metronidazole can be detected by microbiological techniques (bioassays), spectrophotometric methods, thin layer chromatography, gas-liquid chromatography, and HPLC (Turgut and Ozyazici, 2004; Klimowicz *et al*., 2002). Earlier detection methods for metronidazole included bioassays and gas-liquid chromatography; however these methods were time-consuming and did not determine or separate the concentrations of the metabolites. HPLC methods have been

used to determine the concentrations of metronidazole and its metabolites in biological specimens, including urine (Nilsson-Ehle *et al*., 1981), gastric juice (Jessa *et al*., 1996) serum (Galmier *et al*., 1998; Larsen *et al*., 1986, Mattila *et al*., 1983), plasma (Ezzeldin and El-Nahhas, 2012; Klimowicz *et al*., 2002; Emami *et al*., 1996; Robertson *et al*., 1988), saliva (Jessa *et al*., 1996), vaginal tissue (Venkateshwaran and Stewart, 1995), and vaginal fluids (Robertson *et al*., 1988, Larsen *et al*., 1986). It was previously reported that HPLC has a sensitivity to detect metronidazole at the nanogram level per gram of vaginal tissue (Venkateshwaran and Stewart, 1995). As little as 5 ng of metronidazole was detected in serum using this method (Marques *et al*., 1978). The analytical wavelength for detection of metronidazole ranges from 250 nm to 350 nm (Olbrich and Corbett, 2013, Galmier *et al*., 1998).

2.9.3 Pharmacokinetics and bioavailability of metronidazole

Metronidazole is available as oral, intravenous, vaginal and topical formulations (Lofmark *et al*., 2010). The bioavailability of oral metronidazole is approximately 100% (Simms-Cendan, 1996). Several studies have reported the penetration and distribution of metronidazole into various types of tissues and body fluids (Lamp *et al*., 1999). Less than 20% of the circulating metronidazole is bound to plasma proteins (Lofmark *et al*., 2010). Metronidazole is distributed in breast milk with concentrations similar to that found in serum (Simms-Cendan, 1996). Metronidazole has been reported to have good penetration into cerebrospinal fluid (CSF) and the central nervous system (CNS) (Turgut and Ozyazici, 2004; Lamp *et al*., 1999). Drug concentrations in the uterus and fallopian tubes were reported to be 94% and 97.3% of serum concentrations respectively 3 to 4 hours following oral administration (Elder and Kane, 1979 cited by Lamp *et al*., 1999).

Metronidazole is metabolized in the liver into two major metabolites, (1-(2-hydroxy-ethy)-2-hydroxy-methyl-5-nitroimidazole) and 2-methyl-5-nitroimidazole-1- acetic acid (Simms-Cendan, 1996; Turgut and Ozyazici, 2004; Lofmark *et al*., 2010). The acetic acid metabolite which is found in urine does not have any pharmacological activity. Hydroxyl-metronidazole has an anti-microbial potency of approximately 30% that of metronidazole against metronidazole susceptible bacteria and can be detected in blood (Jessa *et al*., 1996; Turgut and Ozyazici, 2004,). The major route of elimination of metronidazole and its metabolites is by renal secretion. Biliary-fecal secretion is minimal (Lofmark *et al*., 2010).

2.10 TYPING OF *Trichomonas vaginalis* ISOLATES

2.10.1 Introduction

Typing of microbes belonging to one species is used as an epidemiological tool to establish lines of transmission of the microbe. This is based on the principle that isolates which are epidemiologically related result from the clonal expansion of a single parent isolate and consequently share characteristics that differ from isolates which are not epidemiologically related (Maslow *et al*., 1993; Arbeit, 1995). Isolates belonging to the same type are referred to as a strain of the species. A characteristic of an organism is useful for typing if it is stable within a strain and diverse within the species. The percentage of typeable isolates, reproducibility, discriminatory power, and ease of interpretation of the test and test results are important criteria for evaluating typing systems (Arbeit *et al*., 1995). Ideally a typing method should be simple, rapid, inexpensive, reproducible and discriminatory. Currently no single typing system fulfils all of these criteria, and therefore different typing methods are used for different organisms and different clinical settings. Typing systems are classified as phenotypic or genotypic (Arbeit *et*

al., 1995).

2.10.2 Phenotyping

Techniques such as biotyping, antibiotic susceptibility testing, serotyping, bacteriophage typing, electrophoretic protein typing with or without immunoblotting and multilocus enzyme analysis are all dependent on phenotypic characteristics for strain differentiation (Maslow *et al*., 1993, Arbeit *et al*., 1995.) These typing methods are often limited by the capability of microorganisms to adjust the expression of such characteristics to environmental circumstances (Maslow *et al*., 1993). Therefore, individual isolates of the same strain can vary phenotypically since some may not express one or more of the characteristics used for typing and as a consequence differ or are non-typeable (Arbeit *et al*., 1995). Approaches to phenotyping of *T. vaginalis* isolates include, but are not limited to, electrophoretic protein typing (Boulos *et al.*, 2012) and multilocus enzyme electrophoresis (Proctor *et al*., 1988)

Electrophoretic protein typing and immunoblotting

Electrophoretic protein typing involves the isolation of whole-cell or cell-surface proteins and separation by SDS-PAGE. The gel is thereafter stained to determine the resultant pattern (Maslow *et al*., 1993). Immunoblotting involves the transfer of the separated products onto a nitrocellulose membrane and then labelling different proteins by means of antisera to certain antigens or with pooled human sera as a source of broadly reactive antibodies (Maslow *et al*., 1993). The bound antibodies can then be detected using commercially available enzyme or fluorescent conjugates. Variations in the proteins profiles can be detected by this method (Maslow *et al*., 1993). Using electrophoretic analysis and immunoblotting Alderete *et al* (1986) detected a minor difference in a distinct protein among three of the four isolates examined. A

recent study identified five different *T. vaginalis* strains among 20 *T. vaginalis* isolates using two dimensional gel electrophoresis without immunoblotting (Boulos *et al*., 2012).

Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MLEE) involves the detection of variations in the electrophoretic mobilities of individual soluble metabolic enzymes. Starch gel electrophoresis is used for the separation of the cellular proteins of microorganisms. Individual enzymes are then detected with the use of specific substrates (Whittam *et al*., 1983, Maslow *et al*., 1993). Using MLEE one study characterised 32 *T. vaginalis* isolates into five groups (Soliman *et al*., 1982). Another study demonstrated the classification of 63 *T. vaginalis* isolates into 15 groups (Proctor *et al*., 1988) using MLEE. This suggests a high discriminatory power for this method.

2.10.3 Genotyping

Genotypic typing techniques have several advantages. These techniques often have high discriminatory power and high reproducibility and can thus provide valuable insight into the epidemiology of pathogens (Van Belkum, 1994). Approaches to genotyping of *T. vaginalis* isolates include, but are not limited to, pulsed field gel electrophoresis (Lehker and Alderete, 1999; Upcroft *et al*., 2006a; Upcroft *et al*., 2009), random amplified polymorphic DNA analysis (Vanacova *et al*., 1997; Snipes *et al*., 2000, Kaul *et al*., 2004; Valadkhani *et al*., 2011; Rukasha *et al*., 2013), and PCR-restriction fragment length polymorphism (Stiles *et al*., 2000; Simões-Barbosa *et al*., 2005; Crucittit *et al*., 2008, Meade *et al*., 2009; Kock *et al*., 2013). More recent DNA-based techniques include multilocus sequence typing that assays single nucleotide polymorphisms (Cornelius *et al*., 2012) and microsatellite genotyping that determines the sizes

of tandem repeats of DNA in the genome (Prokopi *et al*., 2011, Conrad *et al*., 2011).

Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) can separate DNA sequences of up to several megabases in length (Lahiti, 1996). There have been limited reports of the use of PFGE for typing *T. vaginalis* (Lehker and Alderete, 1999; Upcroft *et al*., 2006a; Upcroft *et al*., 2009). Upcroft and colleagues used PFGE together with a selection of gene probes to differentiate between 25 *T. vaginalis* isolates (Upcroft *et al*., 2006a). Of these isolates, five were laboratory isolates from Australia and the United States of America and 20 were clinical isolates from South Africa. The authors suggested that the pyruvate:ferredoxin oxidoreductase B gene probe is suitable for identifying epidemiologically related *T. vaginalis* isolates by PFGE (Upcroft *et al*., 2006a)

Random amplified polymorphic DNA analysis

The random amplified polymorphic DNA (RAPD) method of genotyping includes randomly amplifying segments of the target DNA by using a single primer that has no known homology to the target DNA sequence. Several fragments of various sizes are generated by means of this PCR (Swaminathan and Matar, 1993). RAPD analysis has been extensively used to genotype *T. vaginalis* isolates (Vanacova *et al*., 1997; Hampl *et al*., 2001; Rojas *et al*., 2004; Kaul *et al*., 2004; Jamali *et al*., 2006; Valadkhani *et al*., 2011; Rukasha *et al*., 2013). Studies have demonstrated that RAPD analysis was able to differentiate between *T. vaginalis* isolates obtained from asymptomatic and symptomatic patients (Rojas *et al*., 2004; Kaul *et al*., 2004; Jamali *et al*., 2006; Valadkhani *et al*., 2011). Kaul *et al* (2004) showed that RAPD data

differentiated between *T. vaginalis* isolates from 15 symptomatic and 15 asymptomatic women. Using RAPD analysis Rojas *et al* (2004) reported differences amongst 40 isolates of *T. vaginalis* obtained from symptomatic and asymptomatic women.

Multilocus sequence typing

Multilocus sequence typing (MLST) distinguishes strains of microorganisms using internal sequence fragments of seven housekeeping genes (Aanensen and Spratt, 2005; Maiden, 2006), which are amplified by PCR and sequenced. This method has been used to study populations of prokaryotic and eukaryotic pathogens (Tibayrenc, 2009). A recent study reported that this method was able to distinguish 43 polymorphic nucleotide sites, 51 different alleles and 60 sequence types amongst the 68 *T. vaginalis* isolates assessed (Cornelius *et al*., 2012). It was concluded that MLST is useful for investigating the epidemiology, genetic diversity and population structure of *T. vaginalis* (Cornelius *et al*., 2012). This method seems to have a high discriminatory power.

Microsatellite genotyping

Microsatellites are short sequences of nucleotides organised in tandem repeats usually two to six base pairs in length (Conrad *et al*., 2011). Single nucleotide polymorphisms (SNPs) which are bi-allelic genetic markers can also be used to study variations in DNA sequences (Conrad *et al*., 2011). In order to study genetic diversity of *T. vaginalis*, a panel of 21 microsatellites and six single-copy genes from the *T. vaginalis* genome were identified and validated using seven *T. vaginalis* laboratory strains (Conrad *et al*., 2011). High genetic diversity was observed when these markers were used to genotype over 200 *T. vaginalis* isolates obtained from different geographical locations worldwide (Conrad *et al*., 2012). This method has a high discriminatory

power.

Polymerase chain reaction - restriction fragment length polymorphisms

Polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLP) entails amplifying a known DNA sequence and restriction of the resulting amplicons with restriction endonuclease enzymes. Following gel electrophoresis, the polymorphisms are identified by differences seen in the restriction fragment sizes (Swaminathan and Matar, 1993). This typing method can show minor variations in a gene where a single base variation (point mutation) has created or removed a recognition site for the restriction enzyme (Crucitti *et al*., 2008). PCR-RFLP has been used for typing of *T. vaginalis* (Simões-Barbosa *et al*., 2005; Crucitti *et al*., 2008; Kock et al., 2013).

Crucitti *et al* (2008) used PCR-RFLP with the actin gene as target. They identified eight *T. vaginalis* actin genotypes amongst 151 isolates from the Democratic Republic of Congo and Zambia (Crucitti *et al*., 2008). PCR-RFLP analysis of *T. vaginalis* has also been performed using primers derived from the intergenic spacer (IGS) region of the ribosomal DNA (Simões-Barbosa *et al*., 2005; Kock et al., 2013). No genetic variation was found amongst 60 clinical isolates of *T. vaginals* by IGS–PCR RFLP with the use of eight restriction enzymes (Simões-Barbosa *et al*., 2005). Similarly in SA, Kock and colleagues reported no genetic differences amongst 92 *T. vaginalis* isolates using IGS-PCR RFLP; however they were able to discriminate between the same isolates using RAPD analysis (Kock *et al*., 2013). The discriminatory power of PCR-RFLP is therefore dependent on the region targeted in the genome.

CHAPTER 3

3.1 SPECIMEN COLLECTION AND PROCESSING

This chapter provides a description of the methodology used for specimen collection as well as for culture, maintenance and storage of the *T. vaginalis* isolates.

3.1.1 Specimen collection

Vaginal specimens for culture of *T. vaginalis* were collected from the posterior vaginal fornix during speculum examination, using a dacron swab (Medical Wire and Equipment, UK). Different cohorts of patients were used for the different parts of the study. These cohorts are described in the chapters reporting these studies. The swabs containing the specimens were immediately placed into 15 ml polypropylene conical screw-cap tubes (Evergreen Scientific, USA) containing 5 ml of selective Diamond's trypticase–yeast extract–maltose (TYM) medium (Appendix A). Specimens were transported in this medium at room temperature to the laboratory within 4 to 12 hours from collection depending on the distance between site and laboratory.

3.1.2 Growth requirements

Diamond's TYM medium was used for the culture and maintenance of the organism. The medium was enriched with 10 % heat inactivated horse serum (South African Institute for Medical Research [SAIMR], now National Institute for Communicable Diseases [NICD], Johannesburg, South Africa). Antibiotics were added to inhibit growth of vaginal flora (amikacin [100 mg/ml], ampicillin [1 mg/ml], amphothericin B [5 mg/ml]). Yeast extract was added before the medium was autoclaved, while the antibiotics and horse serum were

added aseptically afterwards. The pH of the medium was adjusted to pH 6. Aliquots of 5 ml of the medium were dispensed into 15 ml polypropylene conical screw-cap tubes (Evergreen Scientific, USA). Prepared media was stored at 4^{o}C and used within 2 weeks of preparation. Quality control of the media was conducted by inoculating the media with *T. vaginalis* reference strain American Type Culture Collection (ATCC) 50138.

3.1.3 Culture

Cultures were performed in the tube in which the specimen was transported. The tubes were placed at 37^{o}C in a 5% CO_2 enriched humidified incubator. The addition of L-cystein in the medium and the low surface/column height ratio created an O_2 gradient, decreasing from top to bottom of the tube, supporting growth of *T. vaginalis* in the O_2 depleted lower regions. The cultures were incubated for seven days. The cultures were examined daily from day 2 to 7. A drop of the culture medium was harvested aseptically from the bottom of the tubes and placed on a glass microscopy slide, covered with a glass coverslip and screened by standard light microscopy at a magnification of 100x and confirmed positive at 450x. A diagnosis of *T. vaginals* infection was considered if cells of approximately 7 to 10 µm in diameter were observed showing the characteristic jerky motility. A culture was deemed negative if no trichomonads were observed during 3 minutes of viewing at day 7. A positive diagnosis was confirmed by an in-house polymerase chain reaction (Chapter 6), targeting a region within the Tv-E650 repeats (Paces *et al*., 1992). Positive specimens were subcultured in Diamond's TYM medium at 72 hour intervals in order to obtain non-contaminated axenic cultures.

3.1.4 Storage

Once non-contaminated axenic cultures were obtained, isolates were subcultured in antibiotic-free Diamond's TYM medium and stored at –70^{o}C. To prepare for storage dimethyl

sulfoxide (DMSO) (Sigma-Aldrich, USA) and inactivated horse serum were added to the *T. vaginalis* culture, to yield a final concentration of 10% and 15% respectively (Procter *et al.*, 1988). The culture tubes were inverted 3 to 4 times to suspend the organisms evenly and to allow them to be coated with horse serum proteins and DMSO. Aliquots of 1 ml of the culture were thereafter distributed into cryovials (Corning®, USA). The cultures were frozen by means of a two-step process by placing them at –20^{o}C for approximately 1 hour followed by transfer to –70^{o}C for long term storage.

3.1.5 Recovery of isolates from storage

Vials of stored culture were removed from the –70^{o}C freezer and placed in a water bath at 37^{o}C for 2 to 5 minutes until thawed. The entire content of the vial was then inoculated into Diamond's TYM medium which was pre-warmed to room temperature. The inoculated media was placed in a 37^{o}C $C0_2$ incubator. After 24 hours, approximately 4 ml of the total of 5 ml medium was siphoned off from the top to remove the DMSO. This was replaced with fresh medium. Wet mount microscopy, as described above (section 3.1.3), was performed after 48 to 72 hours. If viable trichomonads were observed, a subculture was made into fresh antibiotic free medium.

3.2 ETHICAL APPROVAL

The study was approved by the Biomedical Research Ethics Committee (BREC) of the University of Kwazulu-Natal. Ethics approval number: H136/97.

CHAPTER 4

ANTIMICROBIAL SUSCEPTIBILITY TESTING

4.1 INTRODUCTION

Treatment of patients with metronidazole-refractory vaginal trichomoniasis presents a major therapeutic challenge and treatment options are limited (Sobel *et al.,* 2001). Although higher doses of metronidazole are often effective, many patients cannot tolerate this because of serious gastrointestinal discomfort (Cudmore *et al*., 2004). Other patients are allergic to any concentration of the drug (Narcisi and Secor, 1996; Goodhew and Secor, 2013). Metronidazole resistance has been widely reported (Schwebke and Barientes, 2006; Upcroft *et al*., 2009; Bosserman *et al*., 2011; Kirkcaldy *et al*., 2012; Rukasha *et al*., 2013). Recently, *in vitro* metronidazole resistance was detected in 6% of *T. vaginalis* isolates obtained from thirty HIV infected women attending an anti-retroviral clinic in Pretoria, South Africa (Rukasha *et al*., 2013).

In view of these reports of metronidazole resistance and the frequency of intolerance and allergic reactions to the 5-nitroimidazole drugs, alternative therapeutic options are needed (Secor, 2012; Goodhew and Secor, 2013). Several compounds have been tested *in vitro* for efficacy against *T. vaginalis* (Blaha *et al*., 2006; Wright *et al*., 2010; Goodhew and Secor, 2013; Afonso Silva Rocha *et al*., 2014). Miltefosine (approved for treatment of visceral leishmaniasis) and nitazoxanide (approved for use against giardiasis and cryptosporidiosis) have shown efficacy *in vitro* and therefore have the potential to be effective in the treatment of trichomoniasis (Blaha *et al*., 2006; Wright *et al*., 2010; Secor, 2012; Afonso

Silva Rocha *et al*., 2014). Goodhew and Secor (2013) tested 1040 drugs against metronidazole susceptible and resistant strains of *T. vaginalis* and found that none was as effective as any of the 5-nitroimidazole compounds. However the study demonstrated that disulfiram and nithiamide may be effective and could be potentially used to treat individuals with hypersensitivity to 5-nitroimidazole drugs. The authors also showed a synergistic effect of albendazole as well as coenzyme B12 with 5-nitroimidazoles and these may be useful in combination with metronidazole or tinidazole to treat individuals with clinically resistant trichomonas infection (Goodhew and Secor, 2013). Further attempts to identify other suitable drugs to treat *T. vaginalis* infections are still necessary.

This study consists of two parts. The aim of the first part was to test metronidazole and six other antibiotics, some of which are recommended for use in the syndromic management of sexually transmitted diseases (WHO, 2003) for their potential activity against *T. vaginalis*. This was done by standard broth macro-dilution. In the second part of the study a broth micro-dilution method was used (Upcroft and Upcroft, 2001a) to measure the minimum inhibitory concentration (MIC) of metronidazole for a larger number of *T. vaginalis* isolates. In addition, the MIC values obtained were correlated with clinical outcomes in a subset of the symptomatic patients who returned for follow up after receiving treatment according to syndromic management protocol.

4.2 MATERIALS AND METHODS

4.2.1 Susceptibility testing of *T. vaginalis* to syndromic management drugs and anti-protozoal drugs

4.2.1.1 Isolates

A total of 40 *T. vaginalis* isolates from patients in KwaZulu-Natal were included in this part of the study: nine isolates were obtained from females presenting with vaginal discharge syndrome at the STI clinic of the Prince Cyril Zulu Communicable Disease Centre in central Durban and 31 isolates were obtained from female sex workers at a truck stop on the N3 highway in the uThukela district in Kwazulu-Natal. The isolates were grown from vaginal specimens obtained as described in Chapter 3. *T. vaginalis* American Type Culture Collection (ATCC) 50138, a resistant strain with known MIC for metronidazole (Muller *et al*., 1988), was used as control.

4.2.1.2 Preparation of antibiotic stock solutions

The antimicrobial agents used were metronidazole, ciprofloxacin, doxycycline, ceftriaxone, azithromycin, rifampicin and fluconazole. Stock solutions of these antibiotics were prepared by dissolving an appropriate amount of each drug in its respective diluent (Appendix B). The antibiotics as well as their respective solvents and diluents, are shown in Table 4.1. Antibiotic stock solutions were used immediately to perform two fold dilutions or were stored at –20^{o}C in 1 ml aliquots until required. The concentration range tested was 128 – 0.125 µg/ml for all drugs.

Table 4.1: Diluents and solvents of antibiotics used in the broth macro-dilution assay

Antibiotic	Company	Solvent	Diluent
Metronidazole	Sigma-Aldrich, USA	DMSO	Distilled water
Ciprofloxacin	Bayer, Germany	Distilled water	Distilled water
Doxycycline	Pfizer, USA	Distilled water	Distilled water
Ceftriaxone	Roche, United Kingdom	Distilled water	Distilled water
Azithromycin	Pfizer, USA	95% ethanol	Distilled water
Rifampicin	Pfizer, USA	Methanol	Distilled water
Fluconazole	Sigma-Aldrich, USA	Distilled water	Distilled water

4.2.1.3 Inoculum preparation and standardization

Each isolate of *T. vaginalis* was inoculated in duplicate in Diamonds media (Appendix A) and incubated at 37^{o}C in a 5% CO_2 enriched humidified incubator for 48 hours. For the inoculum preparation, 500 µl of *T. vaginalis* culture was added to 5 ml of antibiotic-free Diamonds medium and incubated as above. After 48 hours, the number of viable trophozoites was counted using a haemocytometer (Appendix C). Viability was established by means of trypan blue exclusion (Appendix C). The required inoculum of 1 x 10^4 viable trichomonads per ml was obtained by dilution with antibiotic free Diamond's TYM medium.

4.2.1.4 Drug susceptibility assay

The broth macro-dilution method was used (Ralph *et al*., 1983). Eleven 15 ml polypropylene conical screw-cap tubes (Evergreen Scientific, USA) were used to prepare the dilution series for each antibiotic. A volume of 9 ml of antibiotic-free Diamond's TYM media was added to the first tube, and 5 ml to each to the remaining 10 tubes. One millilitre of antibiotic pre-diluted stock solution (Appendix B) was then dispensed

aseptically into the first tube and mixed with the diluent by vortexing. Two-fold serial dilutions were performed by transferring 5 ml from the first tube to the next and so on. The tubes with antibiotic concentrations not falling within the test range were discarded. Diamond's TYM medium with no antibiotic was used as growth control. An amount of 100 µl of the standardised trichomonad suspension was added to 5 ml of each of the tubes. The tubes were incubated for 48 hours at 37^{o}C in a 5% CO_2 enriched humidified incubator. Assays for each isolate were performed in duplicate.

4.2.1.5 Determination of end points

After 48 hours of incubation, the contents of each tube were mixed by inversion. An aliquot was removed aseptically and viewed microscopically (described in Chapter 3) to determine if the cells showed motility. The end point (MIC) of the assay was the minimum concentration of the drug at which no motile trichomonads were seen. Motility was defined as active movement or stationary cells exhibiting movement of flagella and/or undulating membrane.

4.2.2 Determination of Minimum Inhibiting Concentration of *T. vaginalis* to metronidazole

4.2.2.1 Isolates

One hundred and thirty women presenting with symptoms and signs compatible with vaginal discharge syndrome and 30 asymptomatic women were recruited from those attending the primary health and antenatal clinics at KwaMsane. This is a semi-rural village in the Hlabisa sub-district of Kwazulu-Natal situated within the research area of The Africa Centre for

Health and Population Studies, an approximately 2.5 hour drive north of Durban, Kwazulu-Natal.

The syndromic management protocol for vaginal discharge syndrome which includes metronidazole (2 g single oral dose) was administered immediately to those women presenting with symptoms or on follow-up visit one week later if any of the diagnostic tests were positive in the asymptomatic patients. For the purpose of the study the administration of single dose drugs as well the first dose of multi-dose drugs was observed. Patients were asked to return for follow-up 7 to 10 days after treatment.

4.2.2.2 *T. vaginalis* control strains

Two control strains were included in all assays: one metronidazole resistant strain, STDL/B7268, isolated from a patient in Brisbane who suffered successive treatment failures over two years (Upcroft and Upcroft, 2001a) and a susceptible strain, BRIS/92/STDL/F1623 (Upcroft and Upcroft, 2001a). These strains were kindly donated by Jacqui and Peter Upcroft, Queensland Institute of Medical Research, Brisbane, Australia.

4.2.2.3 Preparation of inoculum

All isolates were grown in Diamond's TYM medium as described in Chapter 3. For the drug susceptibility assays isolates were incubated for 24 hours till the log phase of growth was reached (Upcroft and Upcroft, 2001a). Viable trophozoites were counted in a haemocytometer using trypan blue exclusion (Appendix C). A standard inoculum of 5×10^4 trophozoites was prepared.

4.2.2.4 Drug susceptibility assays

Susceptibility assays were performed as described previously (Upcroft and Upcroft, 2001a). Briefly, a 0.1M (17.1 mg/ml) stock solution of metronidazole (Sigma-Aldrich, USA) in N, N-dimethylformamide (DMF) (HPLC grade, Sigma-Aldrich, USA) was prepared and stored at –20^{o}C. This stock solution was diluted in Diamond's TYM medium resulting in a concentration of 17.1 µg/ml (100 µM) as the highest concentration in the anaerobic assay. A solution of DMF (189.6 µg/ml) without metronidazole was prepared in Diamonds medium and used as growth control. A volume of 200 µl of diluted drug at 2x the final concentration was added to wells 1,3,4,6, etc. of row A and the drug free DMF solution to wells 2, 5, etc. of a 96 well flat bottomed, covered tissue culture plate (Corning-Costar®, USA) and 100 µl of drug free medium was added to all other wells as diluent.

Double dilutions were performed down the plate (Figure 4.1) and the last 100 µl from each well of row H was discarded. The final concentrations of metronidazole after addition of the inoculum in the anaerobic assay ranged from 17.1 µg/ml (100 µM) to 0.1 µg/ml (0.8 µM). The same procedure was followed for the aerobic assay. The final concentrations of metronidazole in this assay ranged from 34.2 µg/ml (200 µM) to 0.3 µg/ml (1.57 µM).

Equal volumes (100 µl) of the required inoculum were added to all wells. For each isolate and drug concentration there were four wells with drug and two without. The plates were placed in the incubation bags and an Anaerocult® C mini sachet (Merck, Germany) was added. The bag was sealed and placed in a regular 37^{o}C incubator. Aerobic assays were placed without bags in the same incubator.

	Isolate A						Isolate B					
Lane	1	2	3	4	5	6	7	8	9	10	11	12
↓ Drug* dilution	M	D	M	M	D	M	M	D	M	M	D	M
Row A (100µM)												
Row B (50µM)												
Row C (25µM)												
Row D (12.5µM)												
Row E (6.25µM)												
Row F (3.12µM)												
Row G (1.6µM)												
Row H (0.8µM)												

*Drug dilution series for anaerobic assay represented M – Metronidazole D – DMF only

Figure 4.1: Diagrammatic representation of the 96-well microtiter test plate

4.2.2.5 Scoring and determination of end points

Using an inverted microscope, trophozoite growth was monitored daily, for up to 72 hours, comparing control and drug containing wells in the same row (Upcroft and Upcroft, 2001a). The anaerobic plates remained in the incubation bags during this procedure. This ensured that there was no fluctuation of the anaerobic environment throughout the incubation period. A standardised scoring system was used to establish the MICs, with scores varying from 1+ to 4+ (Table 4.2).

Table 4.2: Scoring of *T. vaginalis* growth in the broth micro-dilution assay

***T. vaginalis* growth**	**Score**
0-10 motile parasites; not more than 20% coverage of well surface	1+
> 10 motile parasites; 20% to 50% coverage of the well surface	2+
> 50 % coverage of the well surface = almost confluent growth with much motility	3+
confluent growth with full motility	4+

T. vaginalis was monitored for motility and/or percentage well coverage. MIC values were defined as the lowest concentration of drug at which a 1+ score was obtained in at least three out of four of the quadruplicate wells (Upcroft and Upcroft, 2001a)

4.3 RESULTS

4.3.1 Susceptibility testing of *T. vaginalis* to syndromic management and anti-protozoal drugs using broth macro-dilution MIC determination

4.3.1.1 Broth macro-dilution MIC test results

Table 4.3 shows the distribution of the MICs in the 40 isolates. All 40 clinical isolates were susceptible to metronidazole with a MIC range of $\leq$ 0.125 – 0.5 μg/ml. Strain ATCC 50138 displayed the expected MIC >128 μg/ml. As to be expected, ceftriaxone had no inhibitory effect on *T. vaginalis*. No inhibition was observed with fluconazole. While azithromycin and ciprofloxacin had an inhibitory effect, this was only seen at concentrations of 64 μg/ml or higher. Rifampicin showed similar results. The MICs for doxycycline were lower than for any of the other drugs, confirming its effect on protozoa in general.

Table 4.3: Distribution of *T. vaginalis* isolates inhibited at given MIC values (*n* = 40)

Antibiotic	**No of isolates with MIC (µg/ml):**											
	≤ 0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**>128**
Metronidazole	34	5	1	-	-	-	-	-	-	-	-	-
Ciprofloxacin	-	-	-	-	-	-	-	-	-	6	17	17
Doxycycline	-	-	-	-	-	1	12	23	4	-	-	-
Ceftriaxone	-	-	-	-	-	-	-	-	-	-	-	40
Azithromycin	-	-	-	-	-	-	-	-	-	3	27	10
Rifampicin	-	-	-	-	-	-	-	--	1	11	6	22
Fluconazole	-	-	-	-	-	-	-	-	-	-	-	40

The cumulative MICs are shown in Table 4.4. The MIC required to inhibit the growth of 90% of organisms (MIC_{90}) was 0.25 µg/ml for metronidazole, 16 µg/ml for doxycycline, and > 128 µg/ml for ceftriaxone, azithromycin, rifampicin, ciprofloxacin and fluconazole.

Table 4.4: Cumulative MICs of *T. vaginalis* isolates (*n* = 40)

Antibiotic	**Cumulative % of isolates with MIC (µg/ml):**											
	≤ 0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**>128**
Metronidazole	85	97.5	100	-	-	-	-	-	-	-	-	-
Ciprofloxacin	-	-	-	-	-	-	-	-	-	15	57.5	100
Doxycycline	-	-	-	-	-	2.5	32.5	90	100	-	-	-
Ceftriaxone	-	-	-	-	-	-	-	-	-	-	-	100
Azithromycin	-	-	-	-	-	-	-	-	-	7.5	75	100
Rifampicin	-	-	-	-	-	-	-	-	2.5	30	45	100
Fluconazole	-	-	-	-	-	-	-	-	-	-	-	100

4.3.2 Susceptibility testing of *T. vaginalis* to metronidazole using the broth micro-dilution method

4.3.2.1 Metronidazole MICs of *T.vaginalis* isolates with broth micro-dilution

Table 4.5 shows the metronidazole MIC distribution of the 160 isolates obtained with broth micro-dilution under aerobic and anaerobic incubation conditions. In the aerobic assay the MIC of all *T.vaginalis* isolates ranged from 1.1 µg/ml to > 34.2 µg/ml (6.25 µM to > 200 µM) with an average of 9.5 µg/ml (55 µM) after 48 hours of incubation and from 0.3 µg/ml to > 17.1 µg/ml (1.6 µM to > 100 µM) and an average of 2.6 µg/ml (15.2 µM) in the anaerobic assay. After 72 hours the averages were (6.5 µg/ml) (38 µM) and 2.1 µg/ml (12 µM) respectively with identical ranges. The MICs of the controls were within the expected range (Upcroft and Upcroft, 2001a).

Table 4.5: Distribution of metronidazole MICs of 160 *T. vaginalis* isolates incubated aerobically and anaerobically

MIC		**Incubation time and conditions**			
µg/ml	**µM**	**48 hours aerobic incubation**	**48 hours anaerobic incubation**	**72 hour aerobic incubation**	**72 hours anaerobic incubation**
≤ 0.1	**≤ 0.8**	nt	0	nt	0
0.3	**1.6**	0	2	0	11
0.5	**3.13**	0	20	0	25
1.1	**6.25**	1	46	1	55
2.1	**12.5**	8	60	39	51
4.3	**25**	46	19	56	7
8.6	**50**	66	9	47	8
17.1	**100**	35	2	16	2
> 17.1	**> 100**	nt	2	nt	1
34.2	**200**	3	nt	0	nt
> 34.2	**> 200**	1	nt	1	nt

Dilution range – aerobic incubation: 34.2 to 0.3 µg/ml
Dilution range – anaerobic incubation: 17.1 to 0.1µg/ml
nt = not tested

Table 4.6 shows the cumulative % of MICs obtained under anaerobic and aerobic incubation conditions. After 72 hours aerobic incubation the MIC_{90} was 8.6 µg/ml. Under anaerobic incubation, the MIC_{90} was between 2.1 and 4.3 µg/ml.

Table 4.6: Cumulative % of metronidazole MICs of 160 *T. vaginalis* isolates incubated aerobically and anaerobically.

MIC		**Incubation time and conditions**			
µg/ml	**µM**	**48 hours aerobic incubation**	**48 hours anaerobic incubation**	**72 hour aerobic incubation**	**72 hours anaerobic incubation**
≤ 0.1	**≤ 0.8**	nt	0	nt	0
0.3	**1.6**	0	1.3	0	6.9
0.5	**3.13**	0	13.8	0	22.5
1.1	**6.25**	0.6	42.6	0.6	56.9
2.1	**12.5**	5.6	80.1	25	88.8
4.3	**25**	34.4	92	60	93.2
8.6	**50**	75.7	97.6	89.4	98.2
17.1	**100**	97.6	98.9	99.4	99.5
> 17.1	**> 100**	nt	100	nt	100
34.2	**200**	99.5	nt	0	nt
> 34.2	**> 200**	100	nt	100	nt

Dilution range – aerobic incubation: 34.2 to 0.3 µg/ml
Dilution range – anaerobic incubation: 17.1 to 0.1 µg/ml
nt = not tested

4.3.2.2 Response to treatment

Of the 130 symptomatic patients, 56 (43%) returned for follow up after 7 to 10 days of administering of the metronidazole. Thirty two (57%) of these 56 patients were co-infected with HIV. A follow-up specimen was available for 48/56 (86%). Clinical response to treatment was observed in 51/56 (91%) patients. Microbial eradication was seen in 42/48 (88%). No statistically significant difference in clinical response ($p = 0.379$) or microbial eradication ($p = 0.392$) was observed between HIV infected and uninfected women (Table 4.7).

Table 4.7: Clinical response and microbial eradication in HIV negative and HIV positive patients

	HIV +	HIV -	P-value
Clinical Response	88%	96%	0.379
Microbial Eradication	92%	82%	0.392

Table 4.8 and Table 4.9 show the MIC distribution and the cumulative MICs of the 56 pre-treatment isolates of *T. vaginalis*. After 72 hours of aerobic incubation the MIC_{90} was 8.6 µg/ml. Under anaerobic incubation, the MIC_{90} was between 4.3 and 8.6 µg/ml.

Table 4.8: Distribution of metronidazole MICs of 56 pre-treatment isolates of *T. vaginalis* incubated aerobically and anaerobically for 72 hours

MIC		**Incubation conditions**	
µg/ml	**µM**	**Aerobic**	**Anaerobic**
≤ 0.1	**≤ 0.8**	nt	0
0.3	**1.6**	-	2
0.5	**3.13**	0	11
1.1	**6.25**	0	19
2.1	**12.5**	13	13
4.3	**25**	15	3
8.6	**50**	22	5
17.1	**100**	5	2
> 17.1	**> 100**	nt	1
34.2	**200**	0	nt
> 34.2	**> 200**	1	nt

Dilution range – aerobic incubation: 34.2 to 0.3 µg/ml
Dilution range – anaerobic incubation: 17.1 to 0.1 µg/ml
nt = not tested

Table 4.9: Cumulative % of metronidazole MICs of 56 pre-treatment isolates of *T. vaginalis* incubated aerobically and anaerobically for 72 hours

MIC		Incubation conditions	
µg/ml	**µM**	**Aerobic**	**Anaerobic**
≤ 0.1	**≤ 0.8**	nt	0
0.3	**1.6**	0	3.6
0.5	**3.13**	0	23.2
1.1	**6.25**	0	57.1
2.1	**12.5**	23.2	80.3
4.3	**25**	50	85.7
8.6	**50**	89.3	94.6
17.1	**100**	98.2	98.2
> 17.1	**> 100**	nt	100
34.2	**200**	0	nt
> 34.2	**> 200**	100	nt

Dilution range – aerobic incubation: 34.2 to 0.3 µg/ml
Dilution range – anaerobic incubation: 17.1 to 0.1 µg/ml
nt = not tested

4.3.2.3 Association between MIC and response to treatment

Of the patients that, according to the aerobic assay (72 hours), were infected with isolates with respective MICs of ≤ 4.3 µg/ml or 8.6 µg/ml, 24/28 (86%), and 22/22 (100%) responded to treatment. Five of the six patients infected with isolates having MICs of ≥ 17.1 µg/ml responded to treatment as well. The association with MICs obtained with the anaerobic assay read after 72 hours of incubation showed similar associations but at lower MICs. Of the patients infected with an isolate with MIC ≤ 1.1 µg/ml, 28/32 (88%) responded to treatment while for those with isolates with MIC = 2.1 µg/ml and MIC ≥ 4.3 µg/ml the response rates were 13/13 (100%) and 10/11 (91%) respectively. Figure 4.2 (a-b) shows the MIC distribution of pre-treatment isolates of those that responded to syndromic management treatment and those that did not after 72 hours of aerobic and anaerobic incubation. There is a trend towards MICs being lower for the responders. However, the number of non-responders is too small to draw firm conclusions.

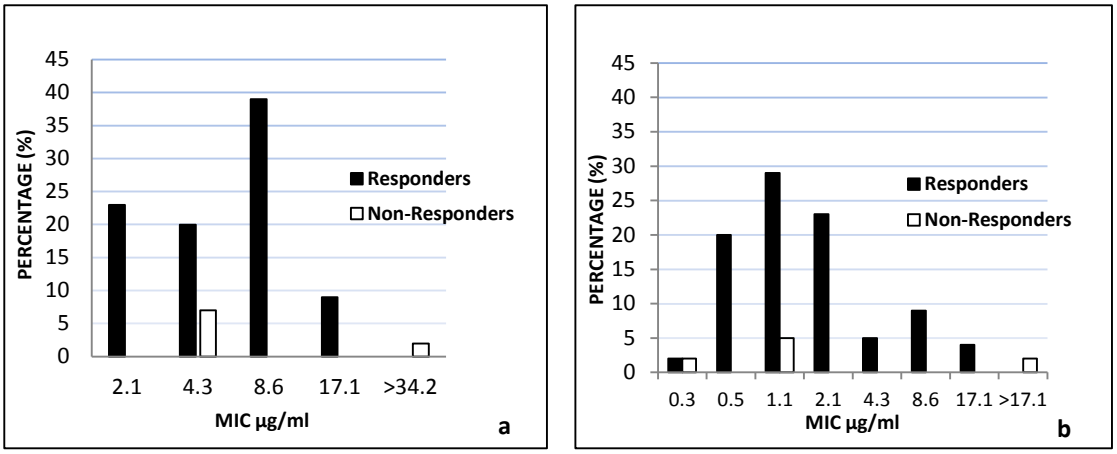


Figure 4.2: MIC distribution *T. vaginalis* isolates of responders and non-responders after 72 hours of aerobic and anaerobic incubation: (a) aerobic incubation; (b) anaerobic incubation.

4.3.2.4 Association between pre-treatment MICs and microbial eradication

Follow up culture results were available for 48 of the 56 patients that came back for follow up. When the Upcroft and Upcroft (2001a) breakpoints were applied, the following was observed: after 72 hours of aerobic incubation, microbial eradication was achieved in 22/24 (92%) of those infected with isolates with MICs of ≤ 4.3 µg/ml, 17/20 (85%) with MICs of 8.6 µg/ml and 3/4 (75%) with MICs of ≥ 17.1 µg/ml. In the anaerobic assay (after 72 hours of incubation), these figures were 24/26 (92%) of the patients infected with a isolate with an MIC of ≤ 1.1 µg/ml, 11/13 (85%) infected with isolates with MICs of 2.2 µg/ml, and 7/9 (78%) with isolates with an MIC of ≥ 4.3 µg/ml. Figure 4.3 (a-b) shows MIC distribution of microbial eradication after 72 hours of aerobic and anaerobic incubation. There is a trend towards MICs being lower in those patients where microbial eradication was achieved. However, MICs of isolates without eradication were evenly distributed within the MIC range.

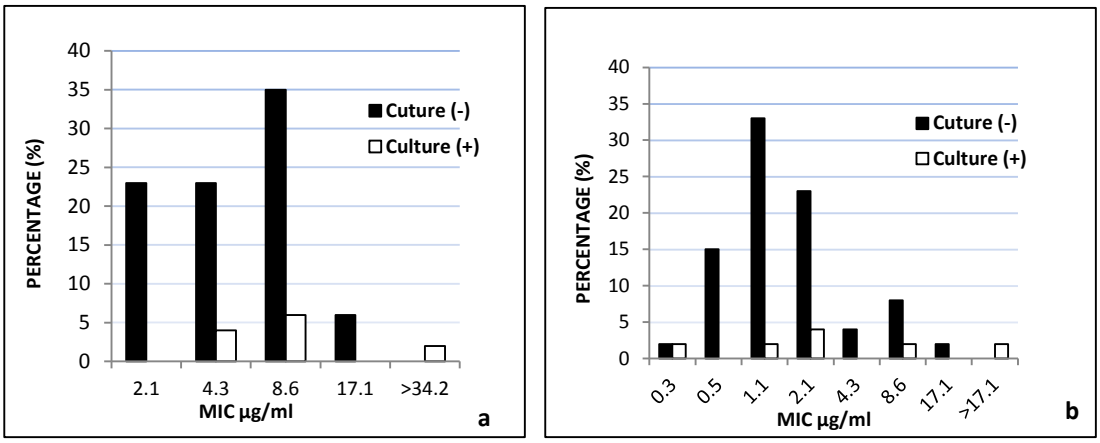


Figure 4.3: Microbial eradication and MIC distribution after 72 hours of aerobic and anaerobic incubation: (a) aerobic incubation; (b) anaerobic incubation.

4.4 DISCUSSION

In the first part of this study, the MICs of 40 *T. vaginalis* isolates and one metronidazole resistant reference strain were performed using the broth macro-dilution method (Ralph *et al*., 1983). At the time of the study, three of the drugs tested (metronidazole, doxycycline and ciprofloxacin), were used as part of the syndromic management package for treatment of vaginal discharge syndrome (Moodley *et al*., 2003). Currently, ceftriaxone has replaced ciprofloxacin (Lewis and Maruma, 2009). These drugs were tested to establish whether there would be additional antimicrobial effect on *T. vaginalis* when used in combination with metronidazole during syndromic management of sexually transmitted diseases. Of the remaining three drugs azithromycin and rifampicin were chosen for their potential effect on protozoal parasites and fluconazole because of the frequent addition of this drug to treatment regimens for vaginal infections.

Azithromycin which has a broad spectrum of antibacterial activity (Davis, 2011) has been used for treatment of infections caused by protozoan parasites, such as *Toxoplasma gondii*

(Nasta and Chiodera, 1997) and *Plasmodium falciparum* (Miller *et al*., 2006). Rifampicin inhibits the prokaryotic RNA polymerase (Campbell *et al*., 2001). This drug could therefore have an inhibitory effect on *T. vaginalis* since this organism shares characteristics with prokaryotic microbes. Azithromycin, rifampicin and ciprofloxacin showed some inhibitory effect but at concentrations which are for bacteria in the resistant range. These three drugs should be tested for synergistic effect with metronidazole.

The results of this study demonstrated that ceftriaxone and fluconazole were not effective *in vitro* when tested against the 40 *T. vaginalis* isolates. This does not exclude a synergistic effect when tested in combination with metronidazole.

Doxycycline is a tetracycline derivative (Tikka *et al*., 2001). These drugs have shown to have anti-protozoal effect, e.g. doxycycline is used for malaria prophylaxis (Tan *et al*., 2011). The results of this study show that doxycycline also affects *T. vaginalis*. Of the 40 isolates 13 (33%) had an MIC $\leq$ 8 μg/ml which is a concentration achievable in serum with an oral dose of 500 mg (Adadevoh *et al*., 1976). To our knowledge there have been no reports on its concentration in vaginal secretions. Whether the MICs in this range translate in contribution to the effect of syndromic management of patients with vaginal discharge syndrome caused by *T. vaginalis* cannot be concluded from this study. Possible synergistic effect of metronidazole with the different tetracycline derivatives should be studied. Of note, one study showed that doxycycline given at 2 x 200 mg/day for 7 days had no therapeutic effect on patients infected with *T. vaginalis* (Mahdi *et al*., 2006). However, only three patients were included and MICs were not performed. With doxycycline MICs ranging between 4 and 32 μg/ml this drug did affect all *T. vaginalis* isolates *in vitro* except

the metronidazole resistant reference strain. This strain was not inhibited by 128 μg/ml. This suggests the presence of a resistance mechanism in *T. vaginalis* for tetracyclines. Whether this is a mechanism leading to resistance to both metronidazole and doxycycline together (as in this strain) warrants further investigation.

Given that there are limited treatment options for trichomoniasis currently, and that clinical failure of 5-nitro-imidazole treatment of trichomoniasis has been reported (Lossick *et al*., 1986; Muller *et al*., 1988; Schwebke and Barientes, 2006; Kissinger *et al*., 2008; Bosserman *et al*., 2011), it is imperative that standardised and reproducible susceptibility tests with good clinical predictive value (Doern and Brecher, 2011) are available.

The problem of assessing *T. vaginalis* resistance to metronidazole is compounded by the lack of universally recognized breakpoints for clinical resistance. Many different methods, reporting on either MICs or minimum lethal concentrations (MLCs), are used to test for drug susceptibility. Furthermore it is not possible to define accurate threshold values for resistant and sensitive strains without correlating outcome with pharmacokinetics, reinfection and MIC and/or MLC.

A variety of breakpoints (Table 4.10) have been used to differentiate between resistant and susceptible *T. vaginalis* isolates with incubation times varying from 24 to 72 hours (Lossick *et al*., 1986; Meri *et al*., 2000; Stiles *et al*., 2000; Upcroft and Upcroft, 2001a; Schwebke and Barrientes, 2006; Kissinger *et al*., 2008; Bosserman *et al*., 2011).

Table 4.10: MIC/MLC breakpoints in broth micro-dilution assays

Classification	Aerobic incubation MIC/MLC Breakpoint		Anaerobic incubation MIC/MLC Breakpoint		Assay incubation period	Reference
	µg/ml	µM	µg/ml	µM		
Susceptible	< 50	< 292	< 3.1	< 18	48 hours	Lossick *et al* (1986)
Resistant	≥ 50	> 292	≥ 3.1	≥ 18		
Susceptible	≤ 75	≤ 439	≤ 15	≤ 88	24 and 48 hours	Meri *et al* (2000)
Resistant	> 75	> 439	> 15	> 88		
Susceptible	< 10	< 58	-	-	24 hours	Stiles *et al* (2000)
Moderate resistance	10–100	58-585	-	-		
High level resistance	≥100	≥ 585	-	-		
Susceptible	≤ 4.3	≤ 25	≤ 1.1	≤ 6.25	48 and 72 hours	Upcroft and Upcroft, (2001a)
Intermediate	8.6	50	2.1	12.5		
Resistant	≥ 17.1	≥ 100	≥ 4.3	≥ 25		
Practically no resistance	< 50	< 292	-	-	46 to 50 hours	Schwebke and Barrientes, (2006)
Very low resistance	50	292	-	-		
Low resistance	100	585	-	-		
Moderate resistance	200	1170	-	-		
High resistance	> 200	> 1170	-	-		
Mild resistance	50–100	292-585	-	-	48 hours	Kissinger *et al* (2008)
Mild-to-moderate resistance	101–199	591-1164	-	-		
Moderate resistance	200–400	1170-2339	-	-		
High resistance	> 400	> 2339	-	-		
Susceptible	< 50	< 292	-	-	48 hours	Bosserman *et al* (2011)
Minimally resistant	50-100	292-585	-	-		
Moderately resistant	200	1170	-	-		
Highly resistant	≥ 400	≥ 2339	-	-		

After comparing *in vitro* test results of isolates from clinical treatment failures and responders, Upcroft and Upcroft, (2001a) proposed aerobic and anaerobic breakpoints, for categorising isolates as susceptible, intermediate and resistant as shown in Table 4.10. Overall, breakpoints proposed by Upcroft and Upcroft (2001a) are much lower in comparison to the other breakpoints listed in Table 4.10.

Table 4.11 shows the classification of the 160 isolates from the second part of the study as susceptible, intermediate or resistant after 48 and 72 hours of incubation according to breakpoints identified by Upcroft and Upcroft, 2001a (Table 4.10). The MICs shifted towards lower readings after 72 hours of incubation in both aerobic and anaerobic environments. According to these breakpoints the MICs obtained after 72 hours under aerobic and anaerobic conditions both displayed metronidazole resistance in approximately 11% of isolates. Four isolates were resistant under both aerobic and anaerobic incubation. Over 50% of isolates were susceptible after 72 hour of incubation under both aerobic and anaerobic incubation. Although the broth micro-dilution method applied here is reproducible, further improvement of the assays may still be necessary. A higher drug dilution range together with an incubation period longer than 72 hours may result in more precise detection of true clinically resistant isolates.

Table 4.11: Interpretation of susceptibility assays using Upcroft and Upcroft (2001a) breakpoints

Number (%) of *T. vaginalis* Isolates (n = 160)						
	Susceptible		**Intermediate**		**Resistant**	
Incubation*	48 hours	72 hours	48 hours	72 hours	48 hours	72 hours
Aerobic	55 (34)	96 (60)	66 (41)	47 (29)	39 (24)	17 (11)
Anaerobic	68 (43)	91 (57)	60 (38)	51 (32)	32 (20)	18 (11)

* row: incubation period; column: incubation environment

Applying the Upcroft and Upcroft (2001a) breakpoints (Table 4.10) to the 56 pre-treatment isolates, 28 (50%) were susceptible and 6 (11%) were resistant to metronidazole after 72 hours of aerobic incubation. The remaining 22 (39%) were inhibited at the concentration in between the two breakpoints. After 72 hours of anaerobic incubation, 32 (57%) of the 56 pre-treatment isolates were susceptible, 11 (21%) were resistant, and 13 (23%) were inhibited at the concentration in between the two breakpoints. Only two isolates were resistant under both aerobic and anaerobic incubation.

Most studies found that high MICs obtained aerobically correlate better with treatment failure (Lossick *et al*., 1986; Muller *et al*., 1988). Our study demonstrated that *in vitro* resistance in clinical isolates occurred under both aerobic and anaerobic conditions. We however did not find a correlation between treatment failure and resistance as only one isolate from a patient with treatment failure was resistant under both incubation environments (Table 4.12).

Based on the MIC breakpoints proposed by Upcroft and Upcroft (2001a), the distribution of MICs, obtained aerobically, in the 56 pre-treatment isolates in this study suggests that approximately 11% of patients diagnosed with trichomoniasis were infected with a resistant strain. This did however not correspond with clinical response, as treatment in only one of six patients with a resistant isolate failed both clinically and microbiologically (Table 4.12).

Table 4.12: Clinical response in metronidazole resistant pre-treatment isolates (n = 6)

Isolate	Aerobic incubation* (72 hours) MIC:		Anaerobic** incubation (72 hours) MIC:		Clinical Response
	µg/ml	µM	µg/ml	µM	
1	17.1	100	2.1	12.5	YES
2	17.1	100	17.1	100	YES
3	17.1	100	1.1	6.25	YES
4	17.1	100	1.1	6.25	YES
5	17.1	100	0.5	3.13	YES
6	> 34.2	> 200	> 17.1	> 100	NO

* Upcroft and Upcroft (2001a) and Stiles *et al* (2000) breakpoints
** Upcroft and Upcroft (2001a) breakpoints

The *T. vaginalis* resistant isolate from this patient displayed an MIC of > 34.2 µg/ml (> 200 µM)) at both 48 hour and 72 hour readings in the aerobic assay and a MIC of > 17.1 µg/ml (> 100 µM) at both 48 hour and 72 hour readings in the anaerobic assay. According to Upcroft and Upcroft (2001a), these MICs represent metronidazole resistance. The only MIC value not found in any of the isolates of the 48 patients that responded to treatment, and in in whom microbial eradication was achieved was > 34.2 µg/ml (> 200 µM), under aerobic incubation. Based on the observations in this patient the Upcroft and Upcroft (2001a) aerobic breakpoint for resistance may be too low.

Susceptibility classification of the 56 pre-treatment isolates varied when each of the breakpoints listed in Table 4.10 were applied for the MICs obtained in the aerobic assay after 72 hours of incubation. As per the breakpoints described by Stiles *et al* (2000), 6/56 (11%) isolates would be categorized as moderately resistant. However, not all of these six isolates (Table 4.12) were part of the eight patients (Table 4.13) with unsatisfactory outcome. Only one of the eight isolates from the latter group (Table 4.13) was amongst the six isolates also classified as resistant according to the Stiles *et al* (2000) aerobic

breakpoint. The other five isolates classified as resistant did respond to treatment. Whether this response is the result of synergistic effect of one or more of the drugs in the syndromic management package or because of inaccuracy of the Stiles *et al* (2000) breakpoint, needs further investigations.

According to the Lossick *et al* (1986), Meri *et al* (2000); Kissinger *et al* (2008) and Bosserman *et al* (2011) breakpoints all 56 isolates would be categorized as susceptible, or having practically no resistance according to Schwebke and Barrientes (2006). This includes all eight isolates of the patients with unsatisfactory outcome.

Table 4.13: Summary of data for patients with unsatisfactory outcome (*n* = 8)

Patient no.	**Aerobic* incubation (72 hours) MIC:**		**Anaerobic* incubation (72 hours) MIC:**		**Clinical Response: (yes/no)**	**Microbiological Eradication: (Culture +/-)**	**Co-infection**
	µg/ml	**µM**	**µg/ml**	**µM**			
1	4.3	25	1.1	6.25	No	Not Done	*Neisseria gonorrhoeae*, syphilis, HIV
2	8.6	50	2.1	12.5	Yes	+	BV (NS = 10)
3	4.3	25	0.3	1.6	No	+	BV (NS = 10)
4	8.6	50	2.1	12.5	Yes	+	BV (NS = 8)
5	4.3	25	1.1	6.25	No	-	BV (NS = 4), yeast, HIV
6	4.3	25	1.1	6.25	No	+	BV (NS = 7), yeast, HIV
7	8.6	50	8.6	50	Yes	+	BV (NS = 7)
8	> 34.2	> 200	> 17.1	> 100	No	+	BV (NS = 9), HIV

* Upcroft and Upcroft (2001a) breakpoints
BV = bacterial vaginosis
NS = Nugent's score

Table 4.13 summarises the data of the eight patients without clinical and/or microbiological response to treatment. Six patients still harboured a *T. vaginalis* isolate on follow-up visit. These observations may be explained by comorbidity. Of the six patients

that were culture positive on follow-up, three reported clinical response while the other three did not. The MICs of the organisms in all eight patients varied from 4.3 μg/ml (25 μM) to > 34.2 μg/ml (> 200 μM) in the aerobic assay and from 0.3 μg/ml (1.6 μM) to > 17.1 μg/ml (> 100 μM) in the anaerobic assay.

A single observed oral dose of metronidazole was administered to all patients in this study, including those that reported for follow-up. Hence, treatment failure cannot be explained by non-adherence. Lack of efficacy of the drug may be based on differences in susceptibility of isolates, on differences in metronidazole pharmacokinetics between patients, early reinfection or non-response to treatment of concomitant infections with other STI pathogens.

In patient 1, no follow up culture was done. The low MICs of the pre-treatment isolate may suggest response to metronidazole treatment and the lack of clinical response could be due to ciprofloxacin resistance in the *N. gonorrhoeae* isolate (Moodley *et al*., 2004). However, both of this is speculation.

Patient 2, 4 and 7 responded clinically but there was no eradication of the *T. vaginalis*. All three also had high bacterial vaginosis (BV) scores. The observation of clinical response of vaginal discharge syndrome is subjective, both by the patient and the clinician. It is therefore possible that these patients' symptoms improved due to the effect of treatment of the BV but that the trichomiasis was still symptomatic although less severe. This means clinical resistance of the *T. vaginalis* while the MICs were low.

Patient 3, 6 and 8 showed no clinical response and there was no eradication of the *T. vaginalis*. All three had a high BV score. The lack of clinical response in these three

patients could have resulted from early recurrence of BV after single dose metronidazole treatment (Moodley *et al*., 2003). However, the presence of yeasts in combination with a Nugent score of 7 in patient 6 could also be compatible with candidiasis (Apalata *et al*., 2014). The persistence of the *T. vaginalis* in patient 8 can also be the result of metronidazole resistance since this isolate showed high MICs.

Patient 5 did not respond clinically but there was eradication of *T. vaginalis*. This patient also had yeasts on pre-treatment microscopy. The absence of response to treatment could be the result of pre-existing candidiasis with colonisation with *T. vaginalis* or the development of candidiasis due to antimicrobial treatment.

Some patients had persistent infection with an organism with a MIC below the Upcroft and Upcroft (2001a) breakpoint for resistance (Table 4.13). It may be possible that these patients were re-infected directly after treatment, or there may have been incomplete absorption of the drug from the gut or poor secretion into the vagina (Kane *et al*., 1961). Like in the gut (van Saene, 1985), there could have also been inactivation of the drug by the vaginal flora. Most resistant isolates were eradicated from our 56 patients by the 2 g single dose of metronidazole. Thus microbiological and/or clinical cure does occur with organisms that have high levels of *in vitro* resistance. As demonstrated in several studies (Lossick *et al*., 1986; Muller *et al*., 1988; Schwebke and Barrientes, 2006; Bosserman *et al*., 2011) including ours, treatment outcome seems not to correlate with *in vitro* susceptibility of the organisms to metronidazole. This needs to be addressed in comprehensive studies that include all variables discussed above.

Table 4.14 shows the comparison between cumulative MICs in the broth macro- and micro-dilution assays in this study. We found that the isolates tested using the broth macro-dilution assay displayed lower MICs compared to the isolates tested using the broth micro-dilution method. Direct comparison of the two assays cannot be made as different methodologies and isolates were used in the assays. Meingassner and Thurner (1979) compared two *T. vaginalis* strains (one metronidazole resistant and one metronidazole susceptible) using the broth macro- and micro-dilution methods. They reported a 32-fold difference in the MLCs of the two strains tested in the aerobic broth micro-dilution assay yet only minor differences were noted between the same two strains in the broth macro-dilution assay. They suggested that the broth micro-dilution method may be a more suitable method for susceptibility testing of the resistant strain since the assay could be conducted under both aerobic and anaerobic conditions ((Meingassner and Thurner, 1979).

Table 4.14: Comparison between the broth macro- and micro-dilution methods

Susceptibility Testing Assays (anaerobic incubation)	**Cumulative % of isolates with MIC (µg/ml):**								
	≤ 0.1	**0.3**	**0.5**	**1.1**	**2.1**	**4.3**	**8.6**	**17.1**	**>17.1**
Broth macro-dilution (48 hour incubation) (Study 1) (*n* = 40)	85	97.5	100	-	-	-	-	-	-
Broth micro-dilution (48 hour incubation) (Study 2) (*n* = 160)	-	1.3	13.8	42.6	80.1	92	97.6	98.9	100
Broth micro-dilution (72 hour incubation) (Study 2) (*n* = 160)	-	6.9	22.5	56.9	88.8	93.2	98.2	99.5	100

Dilution range – broth macro-dilution: 128 to 0.125 µg/ml
Dilution range – broth micro-dilution: 17.1 to 0.1 µg/ml

Comparison of results of published studies on *T. vaginalis* susceptibility is challenging since various methods have been used for testing of metronidazole susceptibility, and results are reported as either MIC or MLC. The differences observed in susceptibility

patterns when applying the different breakpoints (Table 4.10) to our set of susceptibility data underscores the need for the development of globally accepted breakpoints for each method. This would facilitate standardised reporting and direct comparisons of the degree of metronidazole resistance worldwide.

CHAPTER 5

DETERMINATION OF METRONIDAZOLE CONCENTRATION IN VAGINAL SECRETIONS USING A NON-INVASIVE SPECIMEN COLLECTION METHOD

5.1 INTRODUCTION

Clinical failures of metronidazole treatment in women with trichomoniasis have been reported (Muller *et al*., 1988; Schwebke and Barrientes, 2006; Bosserman *et al*., 2011). Several factors such as non-adherence to treatment, poor absorption of the drug from the gastro-intestinal tract, limited secretion into the vagina, inactivation of the drug by vaginal flora, and drug-resistance of *T. vaginalis* ((Kane *et al*., 1961; Kellock and Mahony, 1996; Secor, 1996; Seña *et al*., 2014) may contribute to treatment failure. Although metronidazole has been used for over four decades, very little information is available on the concentration of metronidazole in vaginal secretions after oral administration of the drug (Larsen *et al*., 1986). Several studies (Muller *et al*., 1988; Schwebke and Barrientes, 2006; Bosserman *et al*., 2011), including ours (Chapter 4) have shown poor correlation between *in vitro* resistance of *T. vaginalis* to metronidazole and treatment outcome. Investigating the levels of metronidazole in vaginal secretions will assist in the development of an *in vitro* susceptibility methodology with breakpoints that are better able to predict treatment outcome.

Various specific and sensitive high performance pressure liquid chromatographic (HPLC) protocols have facilitated measuring metronidazole concentration in a variety of biological fluids such as plasma, serum, urine, gastric juice and saliva (Nilsson-Ehle *et al*., 1981, Mattila

et al., 1983, Jessa *et al*., 1996, Emami *et al*., 2006, Nikolin *et al.*, 2004). However, only a few studies have reported on metronidazole concentrations in vaginal secretions using this method (Davis *et al*., 1984; Larsen *et al*., 1986, Robertson *et al*. 1988). These studies utilized various methods to collect vaginal secretions such as cotton swabs (Davis *et al*., 1984; Larsen *et al*., 1986) and sponges (Robertson *et al*. 1988).

The aim of this part of the study is to describe the use of HPLC to measure the time-related secretion of metronidazole in the vaginal secretions of healthy female volunteers using a non-invasive specimen collection method. Concurrent determination of metronidazole in the serum was also undertaken.

5.2 MATERIALS AND METHODS

5.2.1 Subjects

Six healthy female volunteers were recruited from the Nelson R Mandela School of Medicine, University of Kwazulu Natal. These consenting volunteers were given a 2 g single dose of metronidazole orally. Blood specimens were collected at 0, 1, 2, 4, and 8 hours after administration of metronidazole. Vaginal specimens were collected immediately after blood collection at the same time points. After recruitment one volunteer withdrew from the study as she was reluctant to provide blood specimens. Therefore results are presented on specimens of the remaining five volunteers.

5.2.2 Recovery of metronidazole from spiked tampon specimens using an agar diffusion bioassay

To measure the concentration of metronidazole in a spiked tampon specimen, a two-fold serial dilution of metronidazole was made with concentrations in the susceptibility range of the indicator organism. Paper disks loaded with a standard volume of each of these dilutions as well as the fluid expressed from the tampon spiked with a known concentration of metronidazole were placed on the same agar plate seeded with the indicator organism and incubated under suitable conditions.

A stock solution of 10 mg/ml metronidazole (Sigma- Aldrich, USA) was prepared (Appendix B). This stock solution was serially diluted to obtain concentrations ranging from 256 to 32 µg/ml. Sterile 9 mm paper disks were loaded with a 50 µl aliquot of each of these concentrations.

To prepare the spiked tampon specimen, the stock solution was diluted to a final concentration of 320 µg/ml of metronidazole to which 5% foetal calf serum was added. A vaginal tampon (Tampax® regular, Procter & Gamble, UK) was placed into 2 ml of the above solution in a 50 ml tube. Once the solution was absorbed, the tampon was placed in a 30 ml sterile plastic screw–cap container (Sterilin, UK) containing a solution of 10 ml PBS (pH 7). After 1 hour, the tampon was thoroughly expressed using an autoclaved wooden tongue depressor (Sturm *et al*., 2002) and 50 µl of the expressed fluid was added to a sterile 9 mm paper disk.

Bacteroides fragilis (ATCC 25285) was used as the indictor organism. This strain has a metronidazole MIC of 4 µg/ml and is able to form clearly defined inhibition zones at the concentrations used for construction of the standard curve. Cultures of *B. fragilis* were grown and maintained on antibiotic free laked horse blood agar (Appendix D). For the agar diffusion bioassay, a 0.5 McFarland suspension of the organism was evenly distributed on the surface of the laked horse blood agar plate in a Petri dish with a diameter of 90 mm, containing 20 ml of the culture medium.

One disk each with the varying metronidazole concentrations (256 µg/ml, 128 µg/ml, 64 µg/ml and 32 µg/ml) was placed onto the plate. A paper disk containing 50 µl of the tampon fluid was placed in the centre of the plate. The plates were incubated at 37°C for 48 hours under anaerobic conditions using the AnaeroGen atmosphere generation system (Thermo Scientific, USA). This test was performed twice in triplicate. Inhibition zones were read after 48 hours of incubation. The growth inhibition zone diameters (mm) were measured with a calliper. The test was done twice in triplicate.

5.2.3 Measurement of metronidazole concentration in serum and vaginal secretions of volunteers using HPLC

5.2.3.1 Specimen collection and processing

Venous blood was collected from the volunteers before (time 0) and 1, 2, 4, and 8 hours after oral administration of 2 g of metronidazole. The blood specimens were allowed to clot at room temperature ($\pm$ 25^{o}C). The serum was separated from the clot by centrifugation in a clinical centrifuge (10 min at 1500 rpm) and stored at –20^{o}C in 1 ml aliquots.

At each time point, the volunteers were asked to insert a tampon (Tampax® regular, Procter & Gamble, UK) into the vagina immediately after collection of the blood specimens and to remove this after 15-20 minutes of insertion. All volunteers were provided with verbal instructions to ensure correct tampon insertion and removal. After removal, the tampon was placed in a 50 ml sterile plastic screw-cap container (Sterilin, UK) containing 10 ml of sterile distilled water. This was immediately transported to the laboratory and the fluid was harvested as described above in 5.2.2. The vaginal secretions obtained from the tampons were then evaporated to dryness by freeze-drying. Specimens were stored at -20^{o}C until use.

5.2.3.2 High Performance Liquid Chromatography: Instrumentation and conditions

Chromatography was performed with a Spectrochrom Series II (Spectrochrom, UK) single solvent delivery system connected to a variable wavelength Spectrochrom ultraviolet/visible (UV/VIS) detector. The chromatography was performed by reverse phase separation at a flow rate of 2.0 ml/min. The UV/VIS absorbance detector was set at a wavelength of 315 nm, and 1.00 absorbance units. Metronidazole standards and prepared specimens were manually injected via a rheodyne injector with a 10 µl column loop. Chromatographic separation was performed on a RP 18, 55 µm Lichrospher 125 mm cartridge column. (Merck, Germany).

The polar mobile phase was a 70:30 mixture of methanol and potassium dihydrogen phosphate buffer (1.36 g/L). To construct the standard curve, metronidazole (Sigma Aldrich, USA) was dissolved and serially diluted in this mobile phase mixture to produce concentrations of 5 mg, 2.5 mg, 1 mg, 0.5 mg and 0.25 mg metronidazole per 100 ml mobile phase mixture. This enabled the calculation of the metronidazole content of vaginal and serum specimens using an external standard calibration graph. The working range of

standards was selected to target lower concentrations of metronidazole. All reagents and solutions used were either analytical or HPLC grades.

5.2.3.3 Specimen Preparation for HPLC

The freeze-dried vaginal specimens were dissolved in 5 ml of mobile phase solvent. The solution was then filtered through a nylon filter membrane (Sigma–Aldrich, USA) with a pore size of 0.45 µm and the filtrate was injected into the HPLC column.

Preparation of the serum specimens was done based on the method published by Metz *et al* (2002) but modified as follows. After thawing, 1 ml of serum was mixed with 1 ml of cold methanol by vortexing for 20 s. The precipitate was allowed to settle for 10 min. The supernatant was filtered through a nylon filter membrane (Sigma-Aldrich, USA) with a pore size of 0.45 µm and the filtrate was injected into the HPLC column.

5.2.3.4 Data Analysis

Area under the peak was calculated and stored by the Delta Chromatography data system (Digital Solutions Pty. Ltd, Australia). The results were expressed as µg/ml concentrations of metronidazole in serum or vaginal secretion.

5.3 RESULTS

5.3.1 Recovery of metronidazole from a spiked tampon specimen using agar diffusion bioassay

Standard curves for metronidazole were generated by plotting inhibition zone diameter (mm) *versus* log of concentrations (µg/ml). The diameters of growth inhibition zones with

metronidazole reference solutions are shown in Table 5.1. The standard curves (Figure 5.1), demonstrated good linearity in the 32 – 256 µg/ml range.

Table 5.1: Diameters of growth inhibition zones for metronidazole reference solutions obtained from agar diffusion bioassay tests (A and B) for standard curves

	Standards –Test A											
Zone Diameter (mm)	33	31	33.5	29	26.5	29	23	23.5	25	17.5	17	19
Concentration (µg/ml)	256	256	256	128	128	128	64	64	64	32	32	32
Log_{10} Concentration	2.41	2.41	2.41	2.11	2.11	2.11	1.81	1.81	1.81	1.51	1.51	1.51
	Standards –Test B											
Zone Diameter (mm)	37	39	37	32.5	33	33.3	29	29	28.5	21.5	23	23
Concentration (µg/ml)	256	256	256	128	128	128	64	64	64	32	32	32
Log_{10} Concentration	2.41	2.41	2.41	2.11	2.11	2.11	1.81	1.81	1.81	1.51	1.51	1.51

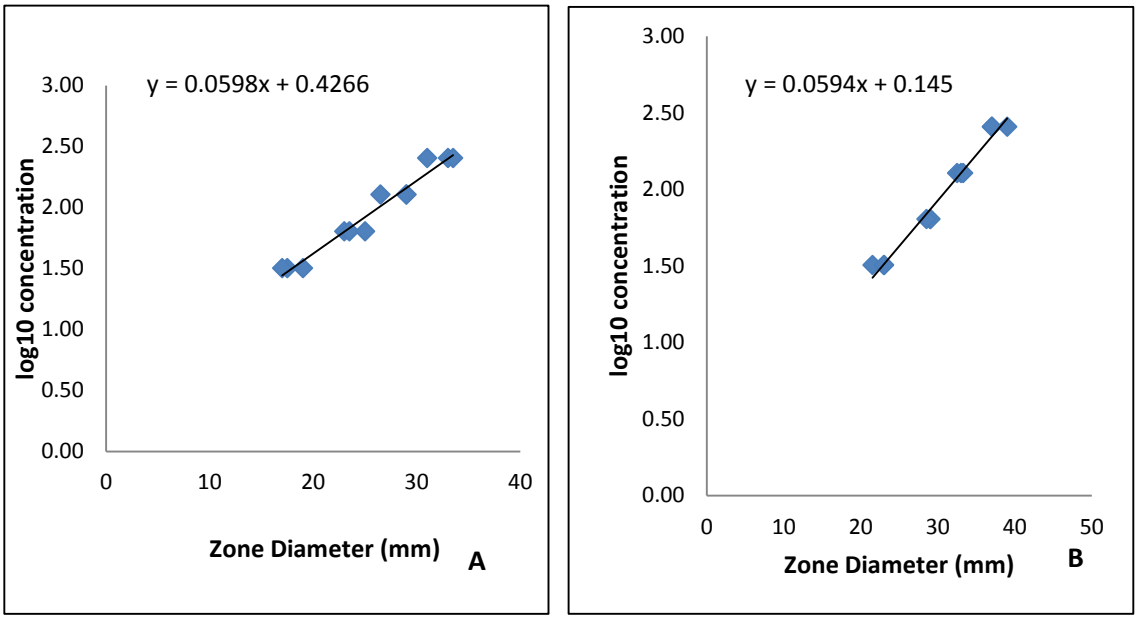


Figure 5.1: Standard curves (Test A and Test B) of log_{10} concentration of metronidazole vs inhibition zone diameter

The representative linear equation for metronidazole was $y = 0.0598x + 0.4266$, in Test A and $y = 0.0594x + 0.145$ in Test B, where x is the zone diameter and y is the $\log_{10}$ of the concentration. The inhibition zones produced by the test specimens are shown in Table 5.2. The average percentage recovery of metronidazole from the tampons spiked with 320 µg/ml metronidazole was 16.7%. This recovery percentage was used to extrapolate the true vaginal concentrations in the volunteers from the measured concentrations.

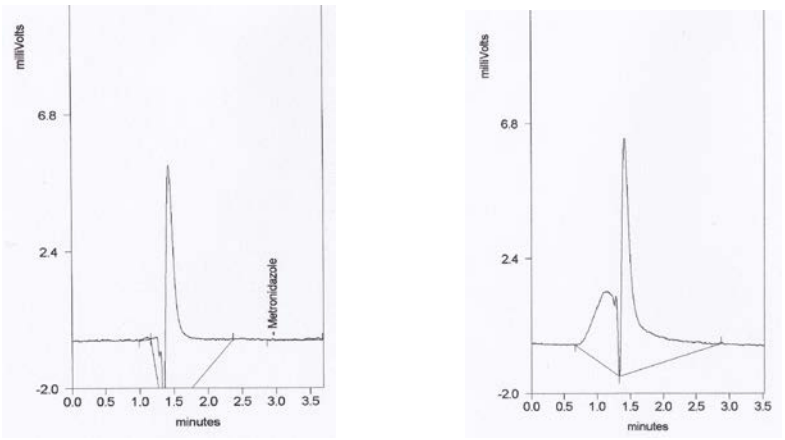
Table 5.2: Concentration (µg/ml) of metronidazole recovered from spiked tampon specimens

	Specimen A				**Specimen B**		
Zone diameter (mm)	23	22	22		26.5	26	25.5
Calculated $\log_{10}$ concentration (µg/ml) concentration	1.80	1.74	1.74		1.72	1.69	1.66
Calculated concentration (µg/ml)	63	55	55		52	49	46

5.3.2 Measurement of metronidazole concentration in serum and vaginal secretions of volunteers using HPLC

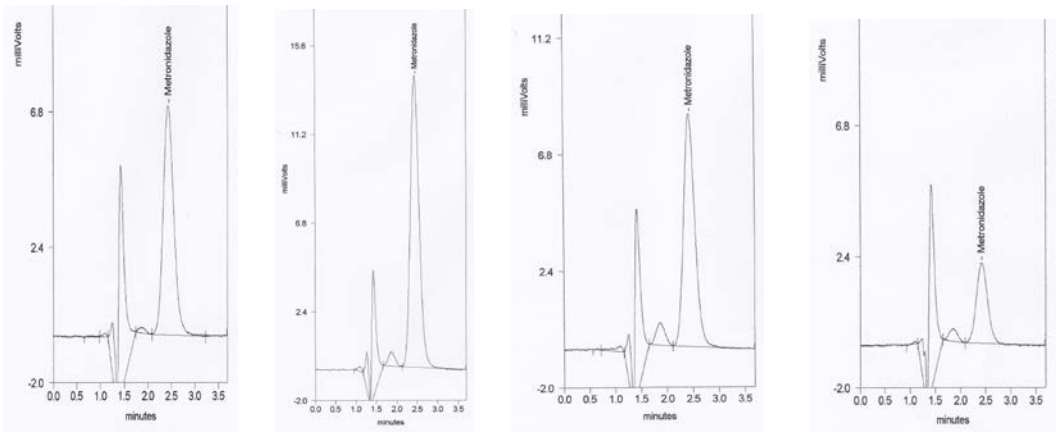
The representative linear equation for metronidazole was $y = 18225.5016x - 32255.67$, where x is the metronidazole concentration and y is the peak area. The chromatograms and standard curve are shown in Appendix E.

No interfering substances were observed in the baseline serum or vaginal specimens collected at 0 hours. Figure 5.2 (a-b) shows chromatograms of a vaginal specimen and serum specimen collected at 0 hours. Figure 5.3 shows chromatograms of serum and vaginal specimens of volunteer C taken at 1, 2, 4 and 8 hours post administration of metronidazole. None of the chromatograms had any interference at the retention time of 2.4 minutes, and the metronidazole peak eluted completely. All chromatograms are shown in Appendix F.



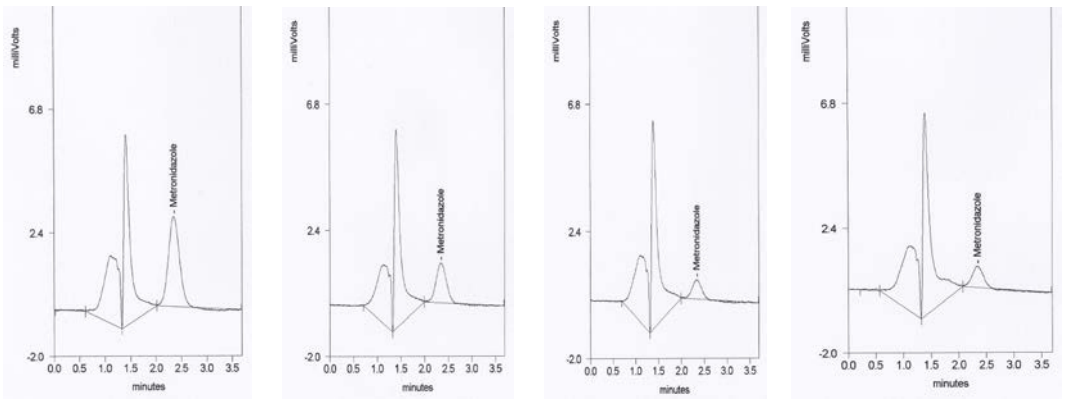
(a) **(b)**

Figure 5.2: Chromatogram of a serum (a) and vaginal secretion (b) obtained from volunteer C after oral administration of metronidazole (2 g) at 0 hours.



1h 2h 4h 8h

(a) Serum



1h 2h 4h 8h

(b) Vaginal secretion

Figure 5.3: Chromatogram of serum (a) and vaginal secretions (b) obtained from volunteer C after oral administration of metronidazole (2 g) at 1, 2, 4 and 8 hours.

The 2 g single oral dose of metronidazole was well tolerated by all five volunteers. Tables 5.3 and 5.4 show the concentrations of metronidazole detected in both vaginal secretions and serum for each of the five volunteers. The data show that all volunteers had a similar absorption pattern, with slight individual variation.

Table 5.3: Serum concentrations of metronidazole in 5 female volunteers after oral administration of 2 g of the drug

Time of specimen collection (hours)	**Concentrations (μg/ml) in volunteer:**							
	A	**B**	**C**	**D**	**E**	**Mean**	**STDev***	**SEM** (±)**
0	0	0	0	0	0	-	-	-
1	6.03	11.81	8.24	9.20	7.15	8.49	2.20	0.99
2	6.99	10.83	13.85	14.34	9.68	11.14	3.04	1.36
4	9.77	11.58	9.11	8.91	9.57	9.79	1.06	0.47
8	7.87	7.63	4.12	7.00	2.97	5.92	2.23	1.00

* Standard deviation
*Standard error of the mean

Table 5.4: Vaginal concentrations of metronidazole in 5 female volunteers after oral administration of 2 g of the drug

Time of specimen collection (hours)	**Concentrations (μg/ml) in volunteer:**							
	A	**B**	**C**	**D**	**E**	**Mean**	**STDev***	**SEM** (±)**
0	0	0	0	0	0	-	-	-
1	0.91	0.74	0.88	0.53	0.67	0.75	0.16	0.07
2	1.19	0.75	0.56	0.96	0.62	0.82	0.26	0.12
4	0.61	0.52	0.47	1.11	1.05	0.75	0.30	0.14
8	0.46	0.60	0.47	0.86	0.56	0.59	0.16	0.07

* Standard deviation
*Standard error of the mean

Maximum metronidazole concentrations in serum and vaginal secretions varied between volunteers between 1 and 8 hours after administration of the drug. The mean measured maximum value in serum was 11.14 µg/ml and 0.82 µg/ml in vaginal secretions. Table 5.5 shows the extrapolated concentrations of metronidazole in vaginal secretions. The level of metronidazole in vaginal secretions varied between 44 and 60% of the amount present in serum (Figure 5.4) with a mean of 51%. The extrapolated mean concentration at 2 hours post administration of the drug was 4.90 µg/ml.

Table 5.5: Extrapolated concentrations (µg/ml) of metronidazole in vaginal secretions

Time of specimen collection (hours)	Volunteer:					Mean	STDev*	SEM (±)**
	A	B	C	D	E			
1	5.46	4.44	5.28	3.18	4.02	4.48	0.94	0.42
2	7.14	4.50	3.36	5.76	3.72	4.90	1.56	0.70
4	3.66	3.12	2.82	6.66	6.30	4.51	1.83	0.82
8	2.76	3.60	2.82	5.16	3.36	3.54	0.97	0.44

Extrapolated metronidazole concentration (µg/ml) in vaginal secretions using a recovery factor of 6

* Standard deviation

* Standard error of the mean

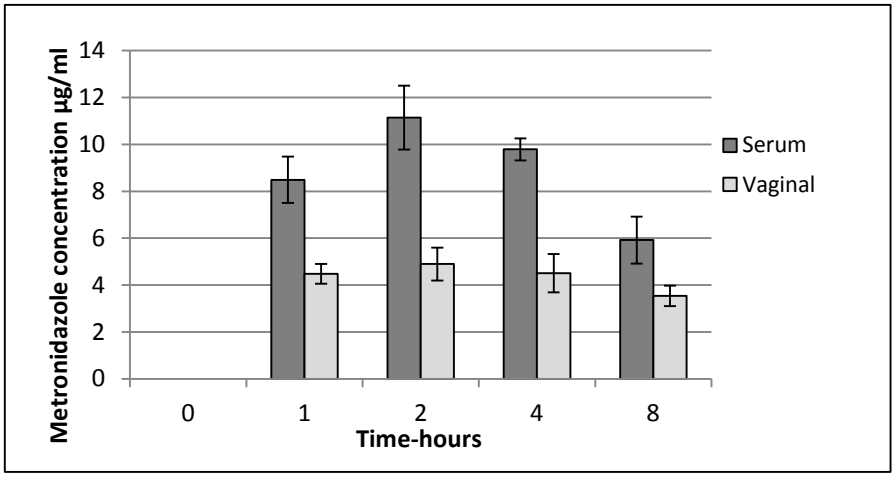


Figure 5.4: Mean concentrations of metronidazole detected in serum (n = 5) and mean extrapolated concentrations in vaginal secretions (n = 5). The bar represents the SEM (±) of the mean serum and vaginal specimen concentrations.

5.4 DISCUSSION

The aim of this part of the study was to demonstrate the feasibility of using a vaginal tampon specimen collection method to determine levels of metronidazole in vaginal secretions using HPLC and to establish the vaginal secretion pattern of metronidazole in women without symptoms of genital tract infection. Concurrent estimation of metronidazole in serum was also done to correlate levels of metronidazole absorption in the blood and secretion in the vagina.

Information currently available on achievable metronidazole concentrations in vaginal secretions is limited. Specimens used for determining metronidazole in the vagina by HPLC have been collected by sponges (Robertson *et al*., 1988) and swabs (Davis *et al*., 1984; Larsen *et al*. 1986) inserted in the vagina by a clinician. Although these various methods of specimen collection resulted in detection of metronidazole, none of these may be practical to be applied to large clinical studies in which specimens need to be collected at time points after the clinical consultation. Self-collection can happen at any time necessary for a study without the women reporting to a health care facility.

We sought to explore the feasibility of using a self-administered-self-collected tampon for collection of vaginal secretions. Given that tampons have a large surface area and are composed of highly absorbent material, substantial amounts of vaginal secretions from the mucosal surface, not limited to a specific area, may be collected using this method. This high absorption capacity may also be a disadvantage as the tampon is able to retain a portion of the biological specimens collected even after processing. In addition, the tampon material may

bind an unknown percentage of the drug. We demonstrated using an agar diffusion bioassay that approximately 83% of the metronidazole was retained in the tampon after processing.

Several studies have described the use of self-administered tampons for specimen collection for diagnosis of STIs including *T. vaginalis*, as well as for bacterial vaginosis (Wilkinson *et al*., 1997; Tabrizi *et al*., 1998; Sturm *et al*., 2002). Tampons were also successfully used to collect cervico-vaginal secretions for detection of endometrial cancer (Fiegl *et al*., 2004). Other studies used medicated tampons to assess the transvaginal absorption of tranexamic acid (Chien *et al*., 1982, Moodley *et al*., 1992). Only one other study reported the use of tampons (Manthei *et al*., 1969 cited by Larsen *et al*., 1986), for collection of vaginal secretions to measure metronidazole concentrations. However highly sensitive methods for detection of metronidazole were not available during that time (Larsen *et al*., 1986). Our study demonstrated that concentrations of metronidazole at the µg level could be detected in vaginal secretions obtained by self-collected tampons using HPLC.

A modification of the method described by Metz *et al* (2002) was used for preparation of the serum specimen for HPLC analysis. Due to the high protein concentration in serum, which would result in HPLC column degradation and plugging, these specimens could not be injected directly onto HPLC columns (Metz *et al*., 2002). Therefore the serum specimen needed to be processed to remove most of the protein prior to injection into the HPLC column. The cold methanol precipitation method and syringe filtration were essential techniques to remove substantial protein concentration (Metz *et al*., 2002).

Apart from anti-protozoal activity, metronidazole and the other nitroimidazoles are potent drugs for the treatment and prophylaxis of infections with anaerobic bacteria.

Pharmacokinetic studies therefore focus on understanding the drug's behaviour in blood and tissues and less on mucosal surfaces. Many studies reported on metronidazole concentrations in the serum after oral administration of the drug (Kane *et al*., 1961; Wood and Monro, 1975; Galmier *et al*., 1998). These form the basis of the pharmacokinetic parameters of metronidazole as summarised in table 5.6. We found median time to maximum concentration of metronidazole in serum to be 2 hours from administration of 2 g of the drug. This confirms that the time till peak concentration (C_{max}) in blood in our study falls within these established parameters (Table 5.6). The C_{max} achieved with 2 g orally in our study is similar to that reported for a 500 mg dose. Two of the three studies with comparable data report higher C_{max} values while one study found levels similar to ours (Table 5.8). This needs further elucidation.

Table 5.6: Pharmacokinetic parameters of metronidazole

Oral absorption	> 90 %
C_{max} 500 mg oral	12 mg/ml after 20 min to 3 h
Plasma half-life	6 - 11 h
Volume of distribution	0.6 - 1.1 l/kg
Plasma protein binding	< 20 %

Edwards, 2003

Since trichomoniasis is mainly a non-invasive infection of the vagina, we tried to establish whether metronidazole levels achievable in vaginal secretions with the standard 2 g oral dose used for treatment of trichomoniasis was comparable with the MIC of *T. vaginalis* isolates in the majority of women responding to treatment. We found that the time of achievement of C_{max} in vaginal secretions followed that in blood with peak levels between 1 and 4 hours. However, the vaginal concentration was on average 51 % of the blood concentration.

Table 5.7: Summary of clinical studies reporting metronidazole concentrations in blood and vaginal secretions

Reference	**Detection method**	**Vaginal specimen collection method**	**Metronidazole dose**	**Mean blood concentration**	**Mean vaginal concentration**
Kane *et al* (1961)	Polarographic method	Not done	200mg orally for 7 days	1 h: 4.8 µg/ml 2 h: 4.5 µg/ml 4 h: 3.7 µg/ml 8 h: 2.9 µg/ml 24 h: 0.8 µg/ml	not done
Wood and Monro (1975)	Thin layer chromatographic method	Not done	2g orally	0.5 h: 35 µg/ml 1 h: 40 µg/ml 2 h: 39 µg/ml 4 h: 38 µg/ml 6 h: 32 µg/ml 24 h: 5.7 µg/ml	not done
Davis *et al* (1984)	HPLC	Cotton swabs	2g orally in one healthy female volunteer	Comparable to concentrations in vaginal secretions	6h: 26.1 µg/ml 12h: 12.6 µg/ml 24h: 6.0 µg/ml 48h: 0 µg/ml
Larsen *et al* (1986)	HPLC	Cotton swab – 1 minute contact	2g orally in 17 patients (including 1 male where seminal fluid was obtained) and 1g (in 30 minutes) intravenously in 7 patients	2 h: 22.9 µg/ml	2h 11.1 µg/ml
Robertson *et al* (1988)	HPLC	Piece of polyester sponge inserted and removed by clinician	High dose metronidazole treatment used: 200mg orally at 07, 15 and 23 hours on days 1 and 2 and then 800mg at the same times on days 3-7.	26h: 8.0 µg/ml	26h: 6.8 µg/ml

A summary of clinical studies reporting metronidazole concentrations in blood and vaginal secretions is presented in Table 5.7. It is impossible to draw conclusions from these studies since the study designs were different. Table 5.8 provides a comparison between the

3 studies in which the metronidazole dose was 2 g and with time of measurement comparable to our study. This shows a wide variation between the reported concentrations.

Table 5.8: Comparison of blood and vaginal concentrations 2 hours post administration of 2 g metronidazole p.o.

Study	**Metronidazole (µg/ml) in:**	
	blood	**vaginal secretion**
Kane *et al* (1961)	4.5	not done
Wood and Monro (1975)	39	not done
Larsen *et al* (1986)	22.9	11.1 (48 %)
This study	11.14	4.90 (44%)

Like in our study, Kane *et al* (1961) also found in human volunteers maximum serum concentrations of metronidazole between 1 and 2 hours of administration of the drug, but at levels half of what we found. This study used polarography in patients infected with *T. vaginalis* on a 200 mg dose of metronidazole three times daily for 7 days. Robertson *et al* (1988) detected comparable concentrations of metronidazole in both plasma and vaginal content, using HPLC, in patients infected with *T. vaginalis*. They used an intra-vaginal sponge to collect the secretions. This is comparable to collection by means of a tampon but the preparation of the specimen for HPLC differed. The higher level of vaginal secretion of the drug could be the result of inflammation of the vaginal wall.

Another study also evaluated metronidazole concentration in vaginal secretions of patients on therapy (Larsen *et al*., 1986) and found, like us, that average vaginal levels were approximately half the serum concentration 2 hours after treatment. They also reported that vaginal levels were comparable to serum levels 6 and 24 hours after treatment. We deduced that concentrations in the vaginal secretions of healthy volunteers were approximately half of

the concentration in serum at all points of measurement up till 8 hours after the drug was administered (Figure 5.4). The lowest concentration of the drug in both serum and vaginal secretions was observed at 8 hours after administration of the drug.

Our findings need to be confirmed by means of a more extensive study in patients with trichomoniasis. This study in healthy volunteers suggests that our approach can be used to investigate, in a non-invasive manner, whether impaired secretion of metronidazole into the vaginal compartment rather than resistance is the cause of failure to respond to standard metronidazole therapy. Based on peak metronidazole absorption patterns observed in serum and vaginal specimens collected from healthy volunteers, it is recommended to measure levels approximately 2 hours after administration of the drug in studies in which metronidazole absorption is measured in patients. The metronidazole secretion patterns may vary in infected individuals.

The level of metronidazole detected in vaginal secretions should be directly related to the MIC breakpoint to be used to predict treatment outcome in infected individuals. Breakpoints currently in use vary from 4.3 to > 400 µg/ml. (Chapter 4, Table 4.10). All but one of these breakpoints are higher than the maximum vaginal concentration observed in the study presented here. However, like the higher breakpoint values, this breakpoint (Upcroft and Upcroft, 2001a) does not differentiate between responders and non-responders to treatment (Chapter 4). Of note, the maximum concentration of metronidazole detected in vaginal secretions was significantly higher (approximately 10 times) than the highest MIC in the broth macro-dilution assay (Chapter 4, table 4.3).

To our knowledge, this is the second report of detection of metronidazole in vaginal secretions using a self-collected tampon specimen. However, the earlier study (Manthei *et al.*, 1969 cited by Larsen *et al.*, 1986) was done > 40 years ago and before the application of sensitive detection methods such as gas liquid chromatography (GLC) or HPLC. Self-collected vaginal tampon specimens evaluated for detection of metronidazole, using HPLC, was successful for the detection of µg levels of metronidazole concentration in vaginal secretions. The preliminary results presented here and the simplicity of this non-invasive specimen collection method and ease of processing suggests that this approach may be useful for investigation of the relationship between treatment failure and metronidazole absorption and vaginal secretion in females with trichomoniasis. This method shows promise for application in future clinical studies.

CHAPTER 6

GENOTYPING OF *T. vaginalis* BY MEANS OF PCR-RFLP

6.1 INTRODUCTION

Molecular typing methods using interspecies variation in the microbial genome to identify subtypes is a valuable tool to study the epidemiology of infectious diseases (Stiles *et al*., 2000). Differentiation of a microbial species into strains may contribute to studies of transmission dynamics. Several molecular typing methods such as PCR-RFLP analysis of genomic DNA (Simões-Barbosa *et al*., 2005; Crucitti *et al*., 2008; Kock *et al*., 2013), RAPD techniques (Vanacova *et al*., 1997; Snipes *et al*., 2000, Kaul *et al*., 2004; Valadkhani *et al*., 2011; Rukasha *et al*., 2013), PFGE (Lehker and Alderete, 1999; Upcroft *et al*., 2006a; Upcroft *et al*., 2009), microsatellite genotyping (Prokopi *et al*., 2011, Conrad *et al*., 2011; Conrad *et al*., 2012; Conrad *et al*., 2013), and MLST (Cornelius *et al*., 2012) have been used previously to differentiate *T. vaginalis* strains.

The discriminatory power of molecular typing systems varies substantially. DNA-based techniques measure fixed, stable differences present in the genome (Stiles *et al*., 2000). The PCR-RFLP method allows detection of point mutations within the sequences of PCR products (Crucitti *et al*., 2008). Mutations are detected by digestion with specific restriction endonucleases (Crucitti *et al*., 2008) followed by gel electrophoresis and staining with ethidium bromide. PCR-RFLP based molecular methods for strain differentiation of *T. vaginalis* using various PCR target regions in the *T. vaginalis* genome have been reported (Stiles *et al*., 2000; Simões-Barbosa *et al*., 2005; Crucitti *et al*., 2008; Kock *et al*., 2013).

One of the explanations for contradictory results of the metronidazole susceptibility test results and response to treatment (Chapter 4) is early reinfection, as reported for gonococcal disease (Moodley *et al*., 2002d). To resolve this, a strain typing method for *T. vaginalis* is needed. In an attempt to develop a genotypic typing method for use in an extension of the work presented here, we targeted a family of 650-bp repetitive sequences in the *T. vaginalis* genome, known as the Tv-E650 repeats (Paces *et al*., 1992). These species-specific Tv-E650 repeats are well conserved in *T. vaginalis* (Paces *et al*., 1992). PCR primers were developed to target a region within these repeats. RFLP analysis of the PCR amplicons was thereafter performed in an attempt to discriminate between strains.

6.2 MATERIALS AND METHODS

6.2.1 Genomic DNA Isolation

One hundred clinical isolates and the reference strain ATCC 50138 (IR 78) were used in this study. This included the 40 isolates used in the broth macro-dilution susceptibility testing (Chapter 4, section 4.2.1). The remaining 60 isolates were fresh isolates obtained from specimens collected from women attending the Africa Centre STI Reference Clinic at KwaMsane in the Hlabisa sub-district of KZN. All specimens were collected and processed as described in Chapter 3.

A haemocytometer was used to count the number of *T. vaginalis* cells per ml (Appendix C) after which the culture was diluted to a final concentration of approximately 10^6 cells/ml. These cells were washed twice in phosphate-buffered saline (PBS), pH 7.4 using centrifugation at 1500 g for 10 min. DNA was extracted from the cell pellets by the addition of 500 µl of lysis buffer followed by incubation at 65^{o}C for 30 min (Shaio *et al.*, 1997). The lysis buffer was

freshly prepared from concentrated stock solutions to obtain final concentrations of 15 mM sodium citrate, 450 mM sodium chloride, 0.2% sodium dodecyl sulfate, and 100 µg of proteinase K per ml. The DNA extracts were purified twice by adding equal volumes of phenol-chloroform (1:1; vol/vol) and once with chloroform only. The aqueous phase was removed by pipetting and the DNA was precipitated with 2 volumes of 95% (vol/vol) ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2). The DNA pellet was washed with 70% (vol/vol) ethanol, air dried, and dissolved in 25 µl of TE buffer (10 mM Tris HCl [pH 7.6], 1 mM EDTA). The preparation of all solutions used is described in Appendix G.

6.2.2 Selection of PCR primers

The two primers used in this study were designed using the published sequences of 650-bp DNA repeats cloned from the *T. vaginalis* genome (Paces *et al*., 1992). One of the members of the repeat sequences, Tv-E650-1, was used to perform a nucleic acid similarity search against the *T. vaginalis* genome using the Blast 2.04 sequence similarity search software (Genbank, http://www.ncbi.nlm.nih.gov/genbank). This yielded eight sequences that were conserved at the 5' and 3' ends (GenBank accession numbers: M86482 to M86489). These sequences were manually aligned and potential primer sets were identified. The specificity of these primers was tested using the Amplify version 2.3 software (Department of Genetics, University of Wisconsin).

For the PCR, a forward primer S1 (5'-TCCCGGATAATTGAAACGGA-3') and a reverse primer S2 (5'-GAATGTGATAGCGAAATGGG-3') were selected to amplify a region of approximately 413-bp within the 650-bp repeat region. The primers were initially synthesized at the University of Cape Town and later by the University of Kwazulu Natal, Molecular Biology Resource Facility.

6.2.3 PCR Amplification

The PCR mixture contained 1 µl d NTPs (10 mM each of dATP, dCTP, dGTP, and dTTP), 20 pmol of each primer, 1 µl template DNA (section 6.2.2), 5 µl of 10x PCR buffer, and 2.5 U AmpliTaq Gold Polymerase (Roche Molecular Systems, United States of America [USA]) in a total volume of 50 µl. The following PCR conditions were used for 40 cycles: 94^{o}C for 45s, 50^{o}C for 40s and 72^{o}C for 1 minute. Final extension consisted of one cycle at 72^{o}C for 7 minutes. PCR amplifications were performed in a Perkin Elmer thermocycler model 9700 (Perkin Elmer Applied Biosystems, USA). With each amplification DNA extracted from the *T. vaginalis* reference strain ATCC 50138 was used as a positive control and distilled water as a negative control.

6.2.4 Agarose gel electrophoresis

Ten microliters of each of the PCR products was electrophoresed on a 1.5 % agarose gel (Appendix G) together with a DNA molecular weight marker XIV (100 base pair ladder) (Boehringer Mannheim Biochemica, Germany) in 1 X Tris/Borate/EDTA (TBE) buffer (Appendix G) at 80 V for 1 hour using a Hoefer PS 500XT DC power supply. The gels were then stained with ethidium bromide (0.5 µg/ml) and viewed under ultraviolet illumination.

6.2.5 RFLP analysis

DNASIS software version 2.1 (Hitachi Software Engineering, Japan) was used to assist in the selection of suitable restriction enzymes. *Acs*1 (Boehringer Mannheim Biochemica, Germany) was selected due to the multiple cleavage sites of this enzyme within the amplicons and thus providing increased probability of discrimination. PCR products were digested according to the

manufacturer's instructions. A volume of 10 µl of the amplicon solution was used for digestion. For the restriction with *Acs*1, the following components were added to an Eppendorf tube on ice in sequence to make up a total volume of 20µl: 7µl sterile distilled water, 2 µl restriction endonuclease buffer, 10 µl amplicon DNA, and 1 µl (10 units) *Acs*1 restriction enzyme.

The components were mixed gently by pipetting, and centrifuged in a microcentrifuge at 12000 x g for 5 seconds before incubating in a water bath for 3.5 hours at 56^{o}C. The reaction was terminated by transferring the tubes immediately on ice after adding 5 µl of gel loading buffer. Twenty microliters of each sample were electrophoresed on a 2 % agarose gel together with a DNA molecular weight marker XIV (100 base pair ladder) (Boehringer Mannheim Biochemica, Germany) in 1 X TBE buffer at 70 V for 1 hour 35 minutes using a Hoefer PS 500XT DC power supply. Restriction fragments were visualised as described above.

6.2.6 Reproducibility and Discriminatory Value of the Typing Method

Reproducibility and stability of the method was established by ensuring that the type strain ATCC 50138 was run as a control with each batch of isolates. The ATCC 50138 isolate was passaged multiple times *in vitro* and separate genomic DNA preparations were made prior to inclusion in each PCR run. The discriminatory ability of the typing method was assessed by using Simpson's Index of Diversity (Hunter and Gaston, 1988; Pillay *et al*., 1998), which calculates the probability that two unrelated isolates within a test population will have different types. A discriminatory value of 0 indicates that all the population members are identical and a value of 1 indicates that all the population members differ from one another. The discriminatory value (D) was calculated according to the formula described in Appendix H.

6.3 RESULTS

PCR on all clinical isolates as well as the reference strain ATCC 50138 produced amplicons of approximately 413-bp in size (Figure 6.1).

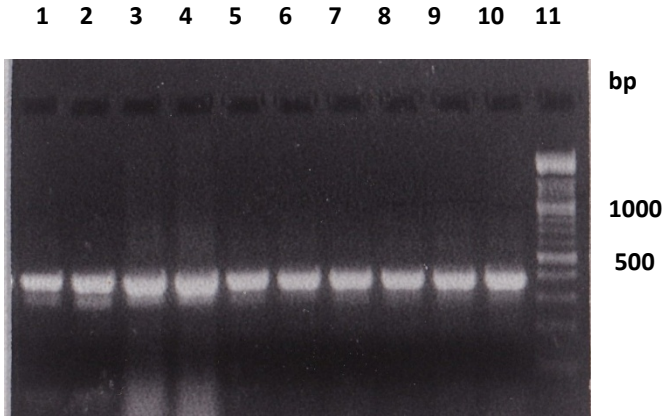


Figure 6.1: Results of the PCR. Lane 1: ATCC 50138; Lanes 2-10: *T. vaginalis* isolates (Lanes 2-10), Lane 11: DNA Molecular Weight Marker XIV

After restriction by means of *Acs1* four patterns were obtained, designated type A to D (Figure 6.2). Only these four types were found among the 100 isolates (Appendix I). The number of bands observed was 4 for type A, 5 for type B, 7 for type C and 6 for type D. The size of the DNA fragments ranged from approximately 400-bp to 100-bp. The type C pattern was predominant and was found in 47/100 isolates. The reference strain also displayed the type C pattern. Type B was found in 28/100, type D in 18/100, and type A in 7/100 isolates. The distribution of the different genotypes is presented in Table 6.1.

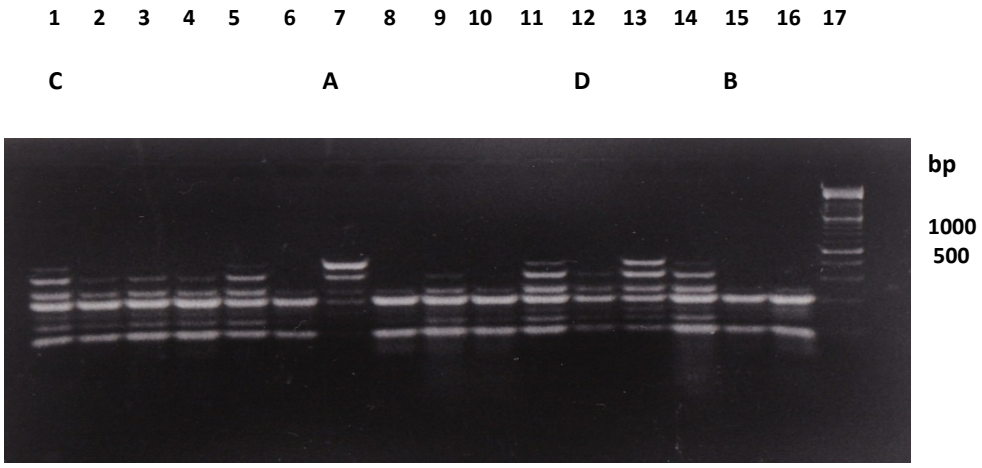


Figure 6.2: *Acs1* RFLP patterns of the PCR amplicon. The designated types of the RFLP patterns are indicated above the lanes. Lanes 1-17: ATCC 50138, KW1, KW2, KW3, STD1, KW4, KW5, KW6, KW7, CSW1, ATCC 50138, KW8, KW9, KW10, KW11, CSW2, DNA molecular weight marker XIV

Table 6.1: Distribution of PCR-RFLP Types (n = 100)

TYPE	**Number (%) of *T. vaginalis* isolates**		
	Africa Centre STI Reference Clinic* (Hlabisa sub-district) (n = 60)	**Female sex worker cohort** (uThukela district) (n = 31)**	**STI Clinic, Prince Cyril Zulu Communicable Disease Centre*** (Durban) (n = 9)**
A	6 (10)	1 (3)	0
B	14 (23)	11 (35)	3
C	25 (42)	17 (55)	5
D	15 (25)	2 (7)	1

The control strain ATCC 50138 was a Type C
*KW, **CSW, ***STD

The PCR-RFLP pattern of the reference strain ATCC 50138 was stable and reproducible. A discrimination index of 0.67 was obtained (Appendix I) using Simpson's Index of Diversity (Hunter and Gaston, 1988).

Table 6.2: Distribution of MICs by genotypes by (n = 41)

Genotype	**MIC (µg/ml)**											
	≤ 0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**>128**
Type A	1											
Type B	12	2										
Type C	19	2	1									1*
Type D	2	1										

*ATCC 50138

Table 6.2 shows the distribution of the MICs of the 40 isolates used in the broth macro-dilution susceptibility testing (Chapter 4, section 4.2.1) according to genotypes. The reference strain ATCC 50138 which had an MIC of >128 µg/ml was a type C. There was no correlation between MIC and genotype. The MICs were distributed across all genotypes.

6.4 DISCUSSION

Genotyping may contribute to the analysis of the relationship between metronidazole susceptibility test results and response to treatment. In addition, molecular typing systems that determine trichomonal diversity could provide valuable insight into the global epidemiology of trichomoniasis.

To be applicable as an epidemiological tool, a typing system needs to be stable and reproducible, to provide a typing result for all isolates within the species and to have sufficient discriminatory power. The PCR-RFLP molecular typing methodology has been shown to provide stable and reproducible results with other organisms (Pillay *et al*., 1998) as well as with *T. vaginalis* (Crucitti *et al*., 2008). We applied this technique as well.

The discriminatory power of molecular typing systems in general varies substantially. For PCR-RFLP this depends on the choice of the nucleic acid target and restriction enzymes. The target sequence for the PCR was chosen because the Tv-E650 repeats are well conserved in *T. vaginalis* strains from different geographical regions and is species-specific (Paces *et al*., 1992). Restriction of the PCR amplicons with *Acs1* produced only 4 types, thus resulting in a moderate discrimination index of 0.67. This indicates that if two *T. vaginalis* strains were randomly selected from a given population, then on 67% of occasions they would fall into different types.

A clonal purification step was not included and we were therefore unable to differentiate infections with mixed parasite lineages. It may be possible that some patients were infected with more than one genotype. Some of the genotype patterns observed could therefore be representative of a mixed infection. On repeated typing, no changes were noted in the PCR products or RFLP pattern of the reference strain ATCC 50138, thus demonstrating both stability and reproducibility. Nonetheless we cannot rule out the possibility that other strains may have been unstable. This however does not impact on the fact that the methodology is moderately discriminative.

The repeat region targeted in this study is well conserved and stable, and therefore may not be variable enough for strain differentiation of STI pathogens in populations with a high transmission rate. In order to establish if isolates are epidemiologically related in short-term transmission studies it is important that target regions used for genotyping have adequate discriminatory power to differentiate among epidemiologically unrelated strains, and also display genetic variation that is stable enough to identify identical types from known sexual contacts (Viscidi *et al*., 2000). This issue has been extensively discussed in studies describing strain typing of *Neisseria gonorrhoeae* (Viscidi *et al*., 2000; Moodley *et al*., 2002d).

Sixty of the 100 isolates were obtained from patients living in the same rural area. Trichomoniasis is endemic in this area with prevalence rates between 30-40% (Sturm *et al*., 1998). Therefore, one would expect a high genetic diversity due to frequent transmission of the organism. Therefore clusters of strains would be expected within a background of strain diversity. This has been shown to be the case for three other endemic STD pathogens e.g. *N. gonorrhoeae* from patients attending the same clinic in KwaMsane (Moodley *et al*, 2002d), as

well as *Haemophilus ducreyi* (Pillay *et al*., 1996) and *Treponema pallidum* (Pillay *et al*., 1998) isolates from the STI clinic at the Prince Cyril Zulu Centre for Communicable Diseases in Durban. This highlights the limitations of the poor discriminatory capacity of the method applied for *T. vaginalis* in this study.

Studies on the correlation between genotype and resistance show contradictory results. Over 50% of our 40 isolates with low MICs (Chapter 4, section 4.2.1) had banding patterns that were identical to that of the metronidazole resistant reference strain ATCC 50138. There was therefore no correlation between genotype and susceptibility testing. A recent study conducted in South Africa also observed no correlation between metronidazole resistance and specific genetic clusters of 30 *T. vaginalis* isolates using a RAPD analysis method (Rukasha *et al*., 2013). Similarly, Rabiee and colleagues reported no correlation with resistance using a PCR method in 15 isolates (Rabiee *et al*., 2012). Genotyping methods invariably use gene targets that are outside the regions that code for drug targets. Therefore, mutations in the genes used for genotyping usually do not correlate with mutations in genes coding for drug targets. This can only be expected if there is clonal spread of a resistant strain (Gandhi *et al*., 2014). It was demonstrated using MLST and microsatellite genotyping that *T. vaginalis* has a two-type population structure (Cornelius *et al*., 2012; Conrad *et al*., 2012). Conrad *et al* (2012) reported a correlation between metronidazole MICs and type 1 and type 2 isolates using microsatellite genotyping. They demonstrated that a higher degree of metronidazole resistance was associated with the type 2 isolates (Conrad *et al*., 2012). Conrad *et al* (2013) also successfully used microsatellite genotyping together with behavioural data, but not MICs, to differentiate treatment failure from reinfection in 42 repeat *T. vaginalis* infections in metronidazole–treated HIV positive women. The reason for this finding could be due to an association between virulence genes and one or more of the microsatellites.

In conclusion, this chapter describes a genotyping system for *T. vaginalis* which has a moderate discriminatory power. This typing method has the ability to differentiate the population of trichomonads investigated in four genotypes. Application of more restriction endonucleases on the same amplicons could improve the system. The PCR-RFLP as described here has the potential to form the basis for more discriminative methodology by combining it with a second typing method with a different target region in the genome. Such an approach was shown to be successful with *T. pallidum* (Pillay *et al*., 1998). Alternatively, a different genotyping method like MLST (Cornelius *et al*., 2012) or microsatellite combined with SNP typing (Conrad *et al*., 2011) may be the solution.

CHAPTER 7

SUMMARY AND DISCUSSION

In **Chapter 1**, a motivation and outline of the study is provided. For a meaningful interpretation of susceptibility testing of *T. vaginalis*, one needs to be able to measure susceptibility to the drugs used for treatment of trichomoniasis quantitatively, to be able to measure the concentration of drugs in the vaginal secretions of patients under treatment, to differentiate between treatment failure and early reinfection and to measure the contribution of other drugs given concomitantly in the context of syndromic management of vaginal discharge syndrome. The study presented in this thesis investigated methodologies that can contribute to answer these questions in one study cohort of patients.

Chapter 2 provides a summary of the literature on the topic, focussing specifically on susceptibility testing of *T. vaginalis* to 5-nitroimidazoles, the mode of action and putative resistance mechanisms, strain typing and metronidazole pharmacokinetics.

Chapter 3 describes the specimen collection method, transport from the clinical sites to the laboratory, the culture technique as well as the preparation of axenic stock cultures and their storage.

In **Chapter 4** two studies on susceptibility testing of *T. vaginalis* are presented. In the first study, broth macro-dilution in Diamond's TYM medium was applied. We tested the effect of antibiotics (metronidazole, doxycycline, ciprofloxacin and ceftriaxone) which are recommended for use in the syndromic management of sexually transmitted diseases to establish whether added antimicrobial effect can be expected when used in combination

with metronidazole. In addition, azithromycin and rifampicin were chosen for their potential activity against *T vaginalis* and fluconazole due to its frequent inclusion in treatment regimens for vaginal infections. All 40 isolates tested were susceptible to metronidazole with MICs ranging from ≤ 0.125 to 0.5 µg/ml. Fluconazole and ceftriaxone demonstrated no *in vitro* activity. Doxycycline had significant inhibitory effect with MICs ranging from 4 to 32 µg/ml. Azithromycin, rifampicin and ciprofloxacin showed inhibitory effect but only at the highest concentrations used. Those drugs that had inhibitory activity on *T. vaginalis* should be investigated further for synergistic effect with metronidazole.

In the second study MICs of 160 clinical isolates were determined using the modified broth micro-dilution method described by Upcroft and Upcroft (2001a). Classification of these 160 isolates using the breakpoints suggested by Upcroft and Upcroft (2001a) showed 11% *in vitro* metronidazole resistance. After 72 hours of incubation, MICs obtained under aerobic incubation ranged from 1.1 µg/ml to > 34.2 µg/ml (6.25 µM to > 200 µM) and from 0.3 µg/ml – > 17.1 µg/ml (1.6 µM to > 100 µM) under anaerobic incubation. Only four isolates were resistant when incubated aerobically as well as anaerobically.

In the 56 (43%) symptomatic patients that returned for follow-up no correlation between *in vitro* resistance and treatment outcome was found. Some patients with isolates classified as susceptible failed to respond to treatment and *vice versa*. Only one patient with an isolate classified as resistant was still culture positive on follow up and failed to respond clinically. Why women infected with susceptible strains of *T. vaginalis* sometimes fail to respond to treatment clinically and microbiologically and why some improve clinically but remain infected with *T. vaginalis* following single dose metronidazole therapy remains unanswered but may possibly be due to the differences in vaginal secretion of

metronidazole between patients or to early reinfection. An additional problem is the lack of uniformity with respect to methodology as well as breakpoints used for resistance.

Chapter 5 describes the use of a self-administered and collected vaginal tampon specimen for measuring of metronidazole concentration in the vaginal secretions of five healthy volunteers, using HPLC. Using an agar diffusion bioassay we demonstrated that there was a 16.7% recovery of metronidazole from the spiked tampon after processing. It was subsequently extrapolated that the metronidazole concentration in vaginal secretions was approximately half the concentration present in serum at each time point. Peak concentrations of metronidazole in serum and vaginal secretions were detected at two hours after oral administration of a 2 g single dose of the drug. It is however important to recognize that due to inflammation of the tissues, the pharmacokinetics of metronidazole in women infected with *T. vaginalis* may differ.

A typing method with high discriminatory power will be required to exclude early reinfection in cases of metronidazole treatment failure. **Chapter 6** describes a PCR-RFLP based genotyping method for characterization of *T. vaginalis* isolates. The primers used were chosen from within a unique family of 650-bp DNA repeats cloned from the *T. vaginalis* genome (Paces *et al*., 1992). Restriction of the PCR amplicons with restriction enzyme *Acs1* differentiated 100 *T. vaginalis* isolates into four types. This method resulted in a moderate discrimination index of 0.67. This PCR-RFLP method did not have the ability to discriminate between resistant and susceptible isolates, but this may be on the account that metronidazole resistance in the population studied was not clonal. Better discriminatory power may be obtained if this PCR-RFLP method is combined with a second typing method targeting a different region in the genome.

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APPENDICES

APPENDIX A

Diamond's TYM Medium

Trypticase (BBL)	20 g
Yeast extract	10 g
Maltose	5 g
L-cysteine	1 g
L-ascorbic acid	0.2 g
Agar	0.5 g
Distilled water	900 ml

The medium was adjusted to a pH of 6.0 and autoclaved for 10 minutes. The medium was then cooled to 48^{o}C and 100 ml horse serum that had been inactivated at 65^{o}C for 30 minutes was added. Thereafter antibiotics were added: 1 g ampicillin, 100 µg/ml amikacin and 5 µg/ml amphothericin B. The medium was stored at 4^{o}C.

APPENDIX B

ANTIBIOTIC STOCK SOLUTIONS

1. Antibiotic stock solutions for broth macro-dilution

A 10x concentration of each antibiotic was prepared as follows:

Final concentration required: 128 µg/ml

In 10 ml media: 128 µg/ml x 10

12800 µg/ml (10x)

Concentration required µg/ml

———————————

Purity µg/ml

12800 µg/ml (x1000)

If purity of antibiotic was 100%: ———————————

1000 µg/ml

12.8 mg/ml

128 mg/10 ml

Antibiotic stock solution 0.128 g/10 ml

2. Metronidazole stock solution for agar diffusion bioassay

Preparation of 10 mg/ml metronidazole stock solution:

0.1 g metronidazole (Sigma-Aldrich, USA) was dissolved in 500 µl acetic acid and made up to 10 ml using autoclaved triple distilled water.

APPENDIX C

Calculation of cell concentration using the haemocytometer

A 1:1 dilution of the cell suspension was prepared by adding 10 µl of cell suspension to 10 µl of 0.4% trypan blue solution (Sigma-Aldrich, USA). Trypan blue differentiates between dead and viable cells. Viable cells appear colourless and dead cells stain blue. Approximately 10 µl of this dilution was added to one of the chambers of the haemocytometer. The cells were viewed under a microscope at 100x magnification. The full grid on the haemocytometer contains a total of 9 squares, each of which is 1 mm^2. The cells were counted on 4 of these 1 mm^2 squares, and the average number of cells was calculated.

The cell concentration was calculated from the following formula:

$$\text{Total cells/ml} = \text{Total cells counted} \times \frac{\text{dilution factor}}{\text{\# of Squares}} \times 10,000 \text{ cells/ml}$$

To determine the number of cells present in the original sample, the cell concentration was multiplied by the total sample volume.

APPENDIX D

Laked Horse Blood Agar

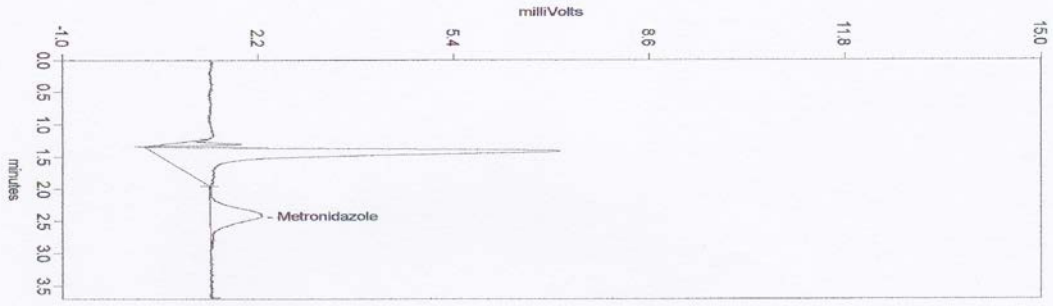
Trypticase peptone	23 g
Glucose	1 g
Yeast extract	2 g
Sodium chloride	5 g
Hemin (1% solution)	10 ml
Vitamin K (1% solution)	1ml
L-cystine	0.5 g
Agar	15 g
Horse blood (laked)	45.5 ml
Distilled water	1000 ml

The powders were weighed and dissolved in 1000 ml distilled water. This solution was autoclaved at 121^{o}C for 15 minutes. The solution was then cooled to 56^{o}C. Hemin (1% solution), vitamin K (1% solution) and horse blood was added. The medium was then dispensed into petri dishes.

APPENDIX E

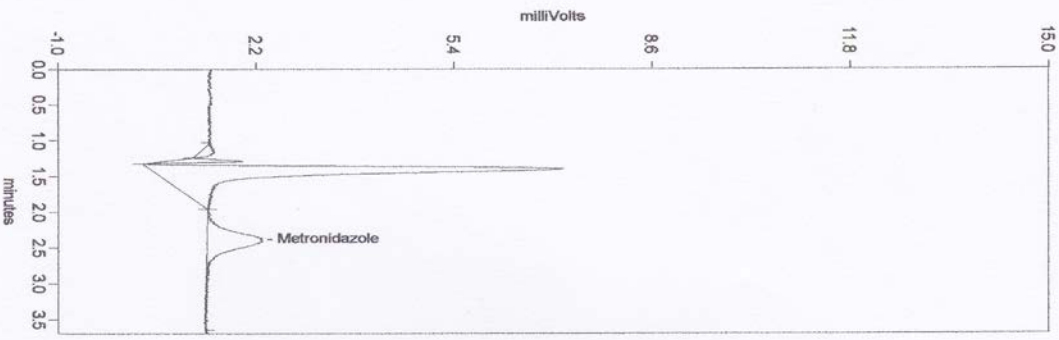
1. HPLC chromatograms of metronidazole standards

```
*******************************************************************************
*                                                                             *
*  TIME :  9:44 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006  *
*                        EXTERNAL STANDARDS REPORT                            *
*                                                                             *
*******************************************************************************

 SAMPLE NAME :   AZOLE:0.25 mg/l-1          CHROMATOGRAM FILE :    AZOLE1-1
 SAMPLE TYPE :   STD1                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME         : METRONID
 DILUTE       :         1.00                INJ                 :  1 OF  2

 Chromatogram AZOLE1-1 recorded at  9:11 am on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



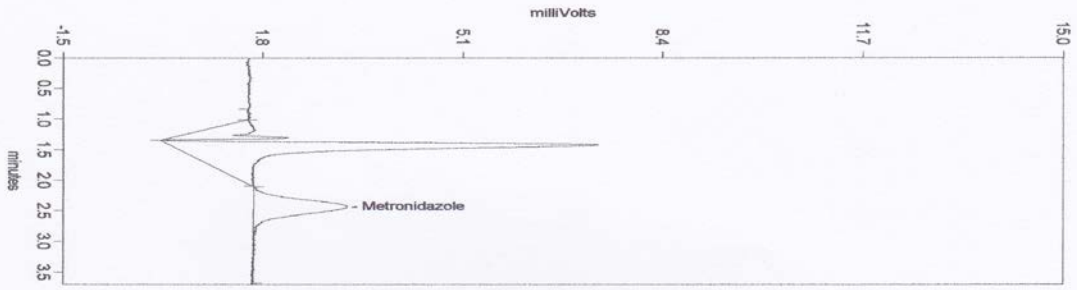
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.42	14.50	14116	0.257
TOTALS :						14116.3	

```
*******************************************************************************
*                                                                             *
*  TIME :  9:45 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006  *
*                        EXTERNAL STANDARDS REPORT                            *
*                                                                             *
*******************************************************************************

 SAMPLE NAME :   AZOLE:0.25 mg/l-2          CHROMATOGRAM FILE :    AZOLE1-2
 SAMPLE TYPE :   STD1                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME         : METRONID
 DILUTE       :         1.00                INJ                 :  2 OF  2

 Chromatogram AZOLE1-2 recorded at  9:17 am on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



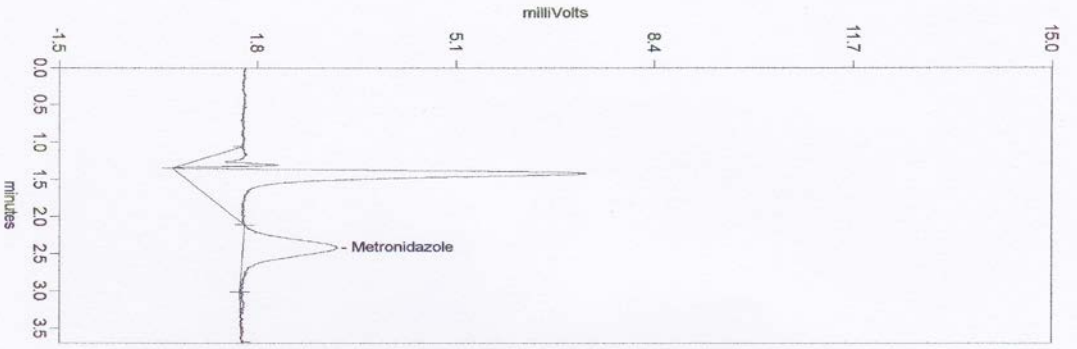
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.36	14.70	15233	0.257
TOTALS :						15232.8	

```
*************************************************************************************
*                                                                                   *
*   TIME :   9:49 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                              EXTERNAL STANDARDS REPORT                            *
*************************************************************************************

 SAMPLE NAME :   AZOLE:0.5mg/l-1          CHROMATOGRAM FILE :    AZOLE2-1
 SAMPLE TYPE :   STD2                     CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  1 OF  2

 Chromatogram AZOLE2-1 recorded at  9:44 am on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



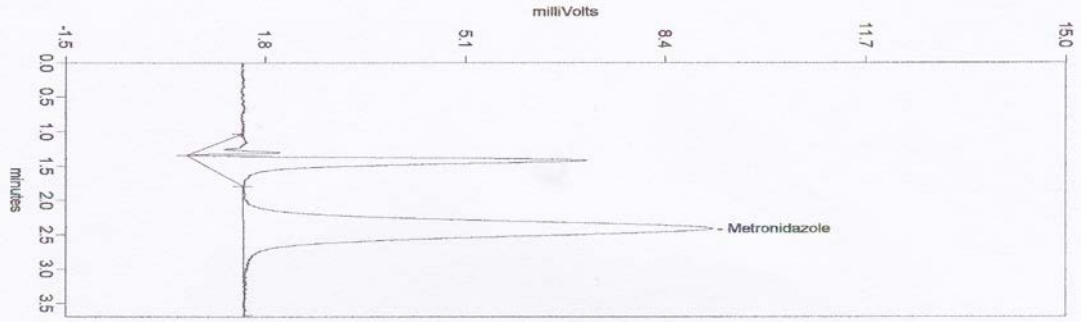
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1	BE	Metronidazol	2.42	14.80	25645	0.491
TOTALS :						25645.5	

```
*************************************************************************************
*                                                                                   *
*   TIME :   9:56 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                              EXTERNAL STANDARDS REPORT                            *
*************************************************************************************

 SAMPLE NAME :   AZOLE:0.5mg/l-2          CHROMATOGRAM FILE :    AZOLE2-2
 SAMPLE TYPE :   STD2                     CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  2 OF  2

 Chromatogram AZOLE2-2 recorded at  9:49 am on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



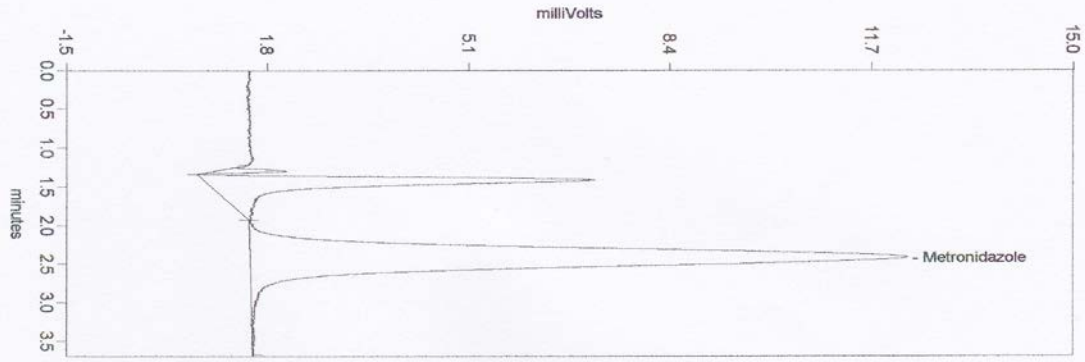
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1	BV	Metronidazol	2.40	14.80	25640	0.491
TOTALS :						25640.3	

```
**********************************************************************************
*                                                                                *
*  TIME : 10:06 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                          EXTERNAL STANDARDS REPORT                             *
**********************************************************************************

 SAMPLE NAME :  AZOLE:1.0mg/l-1          CHROMATOGRAM FILE :   AZOLE3-1
 SAMPLE TYPE :  STD3                     CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00             METHOD NAME         : METRONID
 DILUTE       :         1.00             INJ                 :  1 OF  2

 Chromatogram AZOLE3-1 recorded at  9:56 am on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



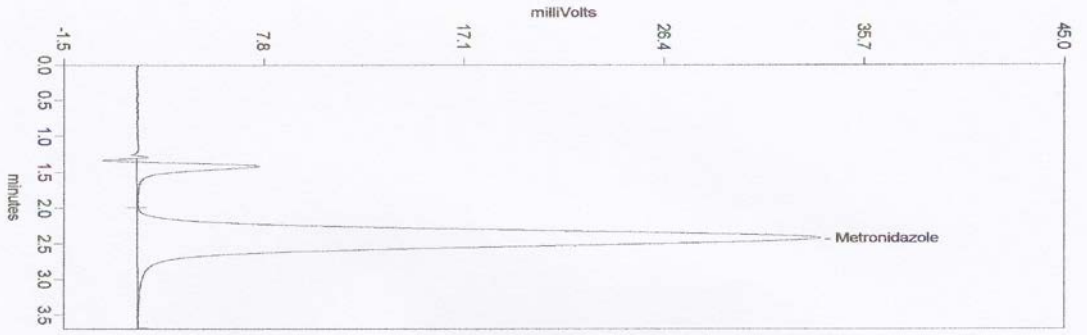
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.40	14.80	129706	1.375
TOTALS :						129705.7	

```
**********************************************************************************
*                                                                                *
*  TIME : 10:11 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                          EXTERNAL STANDARDS REPORT                             *
**********************************************************************************

 SAMPLE NAME :  AZOLE:1.0mg/l-2          CHROMATOGRAM FILE :   AZOLE3-2
 SAMPLE TYPE :  STD3                     CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00             METHOD NAME         : METRONID
 DILUTE       :         1.00             INJ                 :  2 OF  2

 Chromatogram AZOLE3-2 recorded at 10:06 am on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



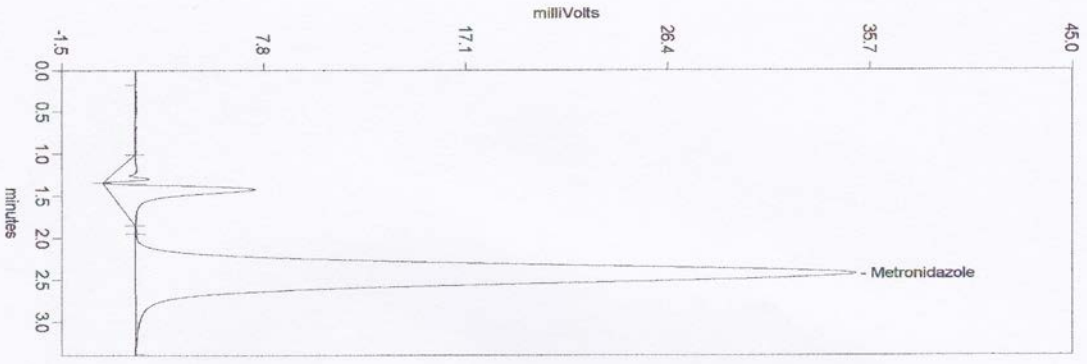
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.41	15.00	181228	1.474
TOTALS :						181227.6	

```
*************************************************************************
*                                                                       *
*  TIME : 12:11 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*************************************************************************

 SAMPLE NAME :   AZOLE:2.5mg/l-1          CHROMATOGRAM FILE :    AZOLE4-1
 SAMPLE TYPE :   STD4                     CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00              METHOD NAME           : METRONID
 DILUTE       :         1.00              INJ                   :  1 OF  2

 Chromatogram AZOLE4-1 recorded at 12:06 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



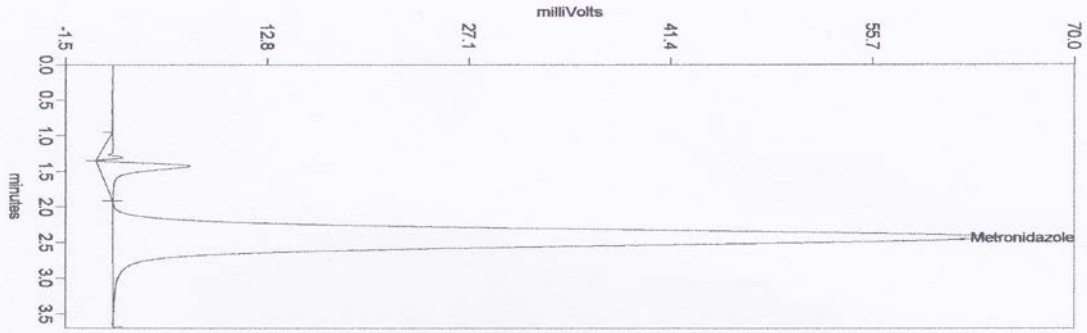
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
2	1		Metronidazol	2.42	15.00	528934	2.620
TOTALS :						528933.9	

```
*************************************************************************
*                                                                       *
*  TIME : 12:17 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*************************************************************************

 SAMPLE NAME :   AZOLE:2.5mg/l-2          CHROMATOGRAM FILE :    AZOLE4-2
 SAMPLE TYPE :   STD4                     CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00              METHOD NAME           : METRONID
 DILUTE       :         1.00              INJ                   :  2 OF  2

 Chromatogram AZOLE4-2 recorded at 12:11 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



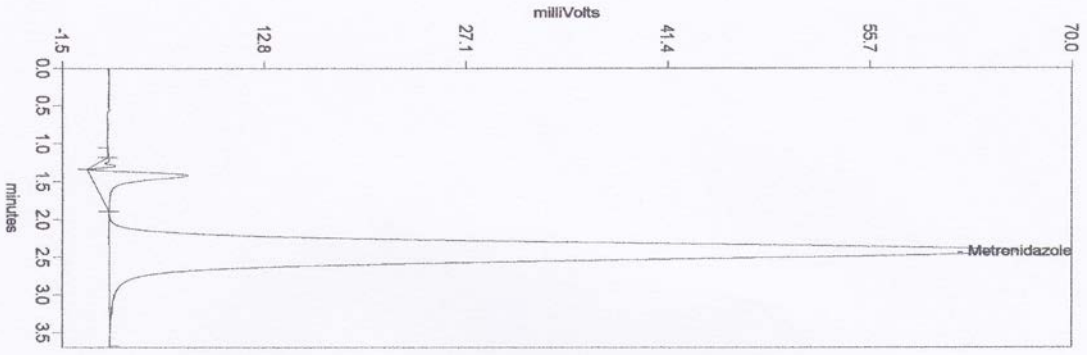
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.43	15.00	550705	2.620
TOTALS :						550705.1	

```
*******************************************************************************
*                                                                             *
*  TIME : 12:23 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006  *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   AZOLE:5.0mg/l-1          CHROMATOGRAM FILE :    AZOLE5-1
 SAMPLE TYPE :   STD5                     CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  1 OF  2

 Chromatogram AZOLE5-1 recorded at 12:17 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.43	15.00	1047251	5.127
TOTALS :						1047251.1	

```
*******************************************************************************
*                                                                             *
*  TIME : 12:28 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006  *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   AZOLE:5.0mg/l-2          CHROMATOGRAM FILE :    AZOLE5-2
 SAMPLE TYPE :   STD5                     CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  2 OF  2

 Chromatogram AZOLE5-2 recorded at 12:23 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.42	14.90	1069461	5.164
TOTALS :						1069461.1	

2. Metronidazole standard curve

Table 1: Average peak areas for metronidazole working standard range

Metronidazole concentration (µg/ml)	Average peak standard area
2.575	14675
5.1500	25643
10.3000	155467
25.7500	539820
51.5000	1058356

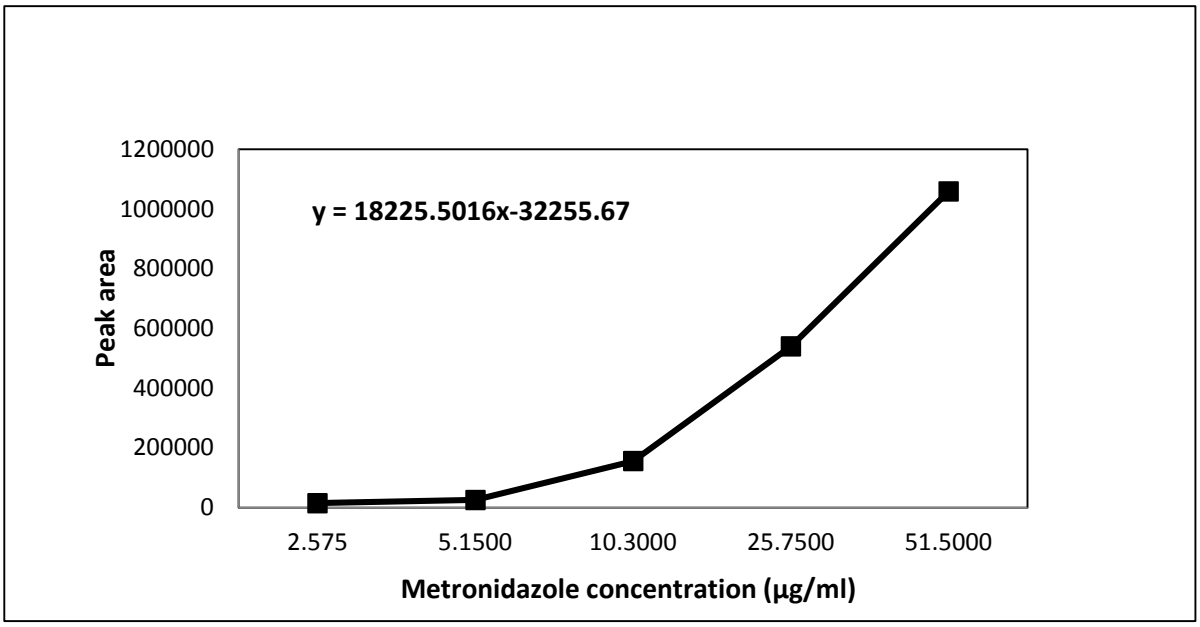


Figure 1: Metronidazole standard calibration curve

APPENDIX F

1. HPLC chromatograms of biological specimens

Vaginal Secretions

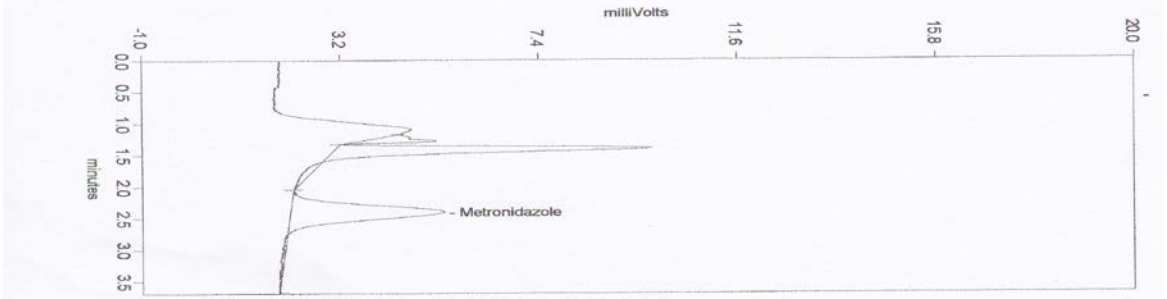
Volunteer A

TIME : 4:29 pm DELTA CHROMATOGRAPHY DATA SYSTEM DATE : 8/12/2006
EXTERNAL STANDARDS REPORT

SAMPLE NAME : Vaginal Tampon-1
SAMPLE TYPE : SAMP
SAMPLE AMOUNT : 1.00
DILUTE : 1.00

CHROMATOGRAM FILE : VGAC01-1
CHROMATOGRAM SOURCE : ACQUIRE
METHOD NAME : METRONID
INJ : 1 OF 2

Chromatogram VGAC01-1 recorded at 4:24 pm on 08/12/2006
Captured by analyst TLN using method : METRONID
Metronidazole content:mg/l



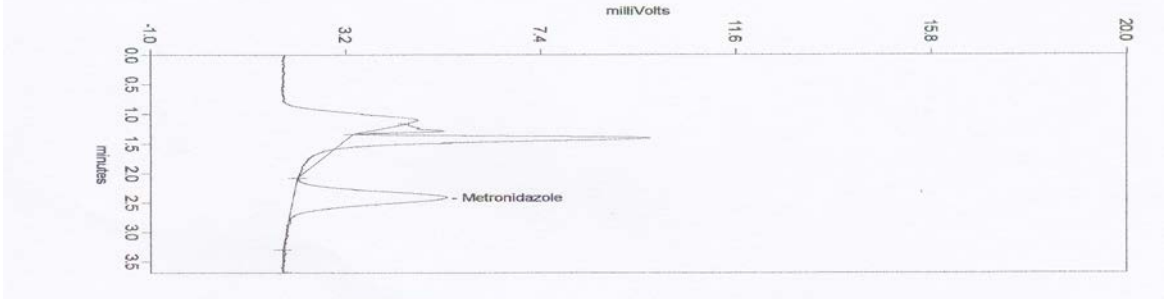
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.40	14.40	48756	0.048
TOTALS :						48756.4	

TIME : 4:35 pm DELTA CHROMATOGRAPHY DATA SYSTEM DATE : 8/12/2006
EXTERNAL STANDARDS REPORT

SAMPLE NAME : Vaginal Tampon-2
SAMPLE TYPE : SAMP
SAMPLE AMOUNT : 1.00
DILUTE : 1.00

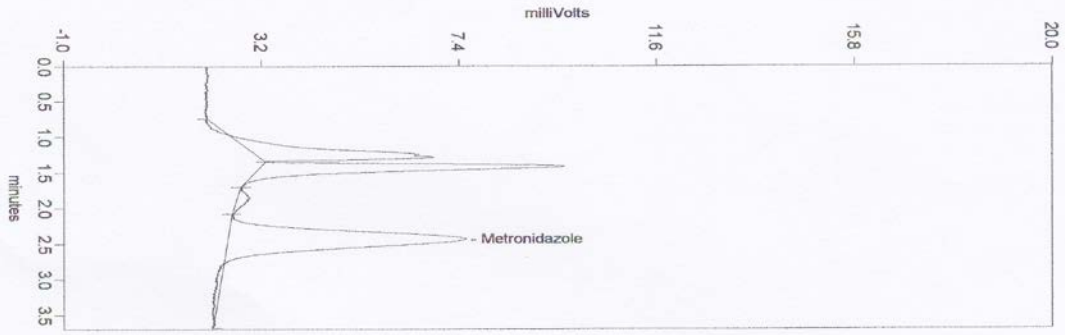
CHROMATOGRAM FILE : VGAC01-2
CHROMATOGRAM SOURCE : ACQUIRE
METHOD NAME : METRONID
INJ : 2 OF 2

Chromatogram VGAC01-2 recorded at 4:29 pm on 08/12/2006
Captured by analyst TLN using method : METRONID
Metronidazole content:mg/l



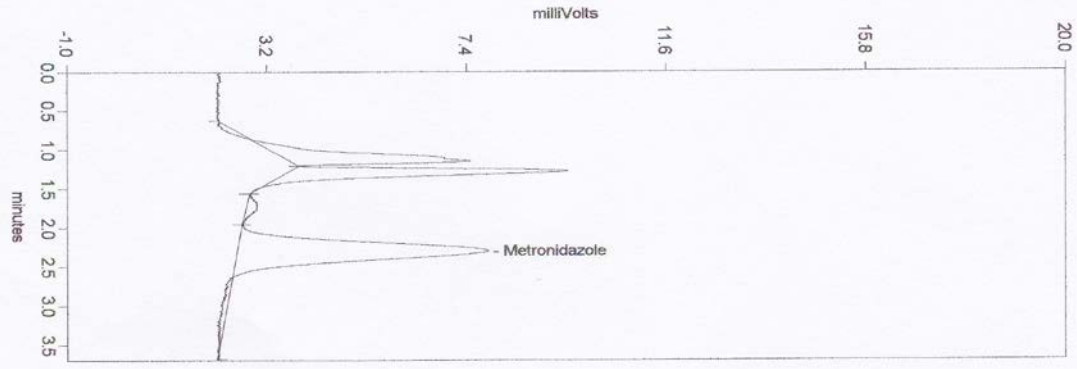
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.41	14.40	52760	0.052
TOTALS :						52760.3	

```
*************************************************************************
*                                                                       *
*  TIME :  4:11 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                            EXTERNAL STANDARDS REPORT                  *
*************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGAC02-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  1 OF  2

 Chromatogram VGAC02-1 recorded at  4:05 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.42	14.50	75008	0.074
TOTALS :						75007.6	

```
*************************************************************************
*                                                                       *
*  TIME :  4:18 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                            EXTERNAL STANDARDS REPORT                  *
*************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGAC02-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  2 OF  2

 Chromatogram VGAC02-2 recorded at  4:11 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



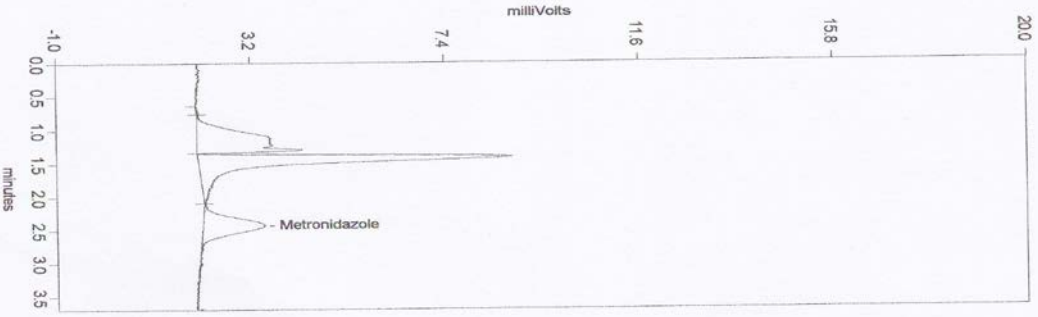
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.29	14.50	77858	0.077
TOTALS :						77857.8	

TIME : 4:01 pm DELTA CHROMATOGRAPHY DATA SYSTEM DATE : 8/12/2006
EXTERNAL STANDARDS REPORT

SAMPLE NAME : Vaginal Tampon-1
SAMPLE TYPE : SAMP
SAMPLE AMOUNT 1.00
DILUTE : 1.00

CHROMATOGRAM FILE : VGAC04-1
CHROMATOGRAM SOURCE : ACQUIRE
METHOD NAME : METRONID
INJ : 1 OF 2

Chromatogram VGAC04-1 recorded at 3:55 pm on 08/12/2006
Captured by analyst TLN using method : METRONID
Metronidazole content:mg/l



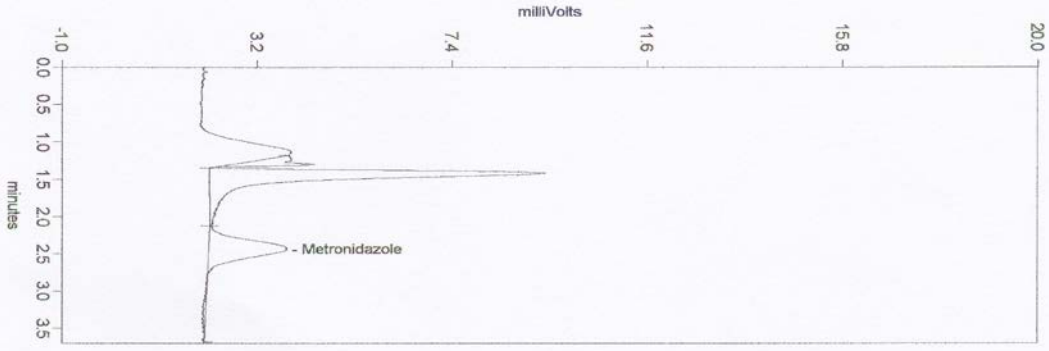
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.42	14.70	21286	0.021
TOTALS :						21286.1	

TIME : 4:05 pm DELTA CHROMATOGRAPHY DATA SYSTEM DATE : 8/12/2006
EXTERNAL STANDARDS REPORT

SAMPLE NAME : Vaginal Tampon-2
SAMPLE TYPE : SAMP
SAMPLE AMOUNT 1.00
DILUTE : 1.00

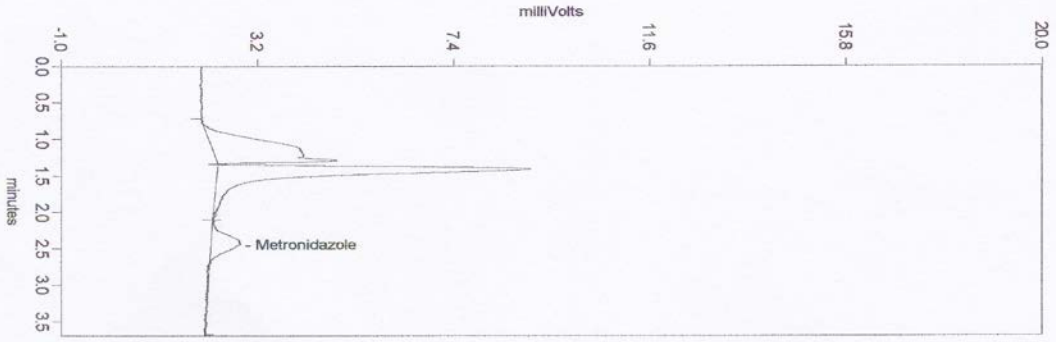
CHROMATOGRAM FILE : VGAC04-2
CHROMATOGRAM SOURCE : ACQUIRE
METHOD NAME : METRONID
INJ : 2 OF 2

Chromatogram VGAC04-2 recorded at 4:01 pm on 08/12/2006
Captured by analyst TLN using method : METRONID
Metronidazole content:mg/l



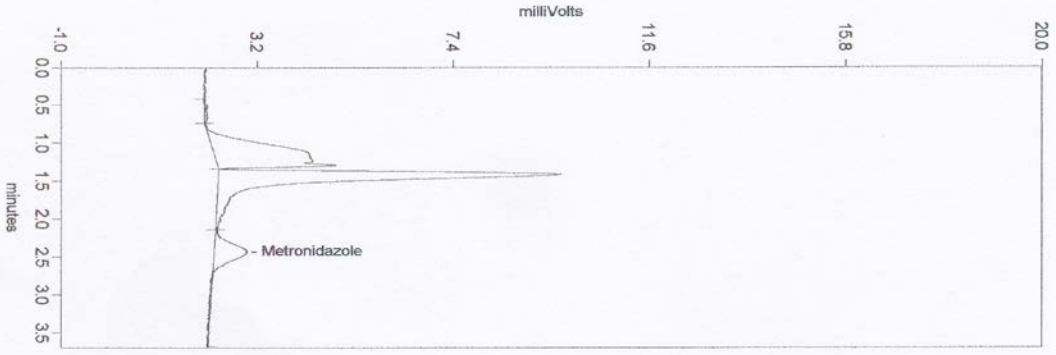
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.43	14.50	25105	0.025
TOTALS :						25105.2	

```
*************************************************************************
*                                                                       *
*  TIME :  3:51 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                            EXTERNAL STANDARDS REPORT                  *
*************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGAC08-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT         1.00               METHOD NAME         : METRONID
 DILUTE        :       1.00               INJ                 :  1 OF  2

 Chromatogram VGAC08-1 recorded at  3:45 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.44	14.20	9046	0.009
TOTALS :						9046.0	

```
*************************************************************************
*                                                                       *
*  TIME :  3:55 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                            EXTERNAL STANDARDS REPORT                  *
*************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGAC08-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT         1.00               METHOD NAME         : METRONID
 DILUTE        :       1.00               INJ                 :  2 OF  2

 Chromatogram VGAC08-2 recorded at  3:51 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



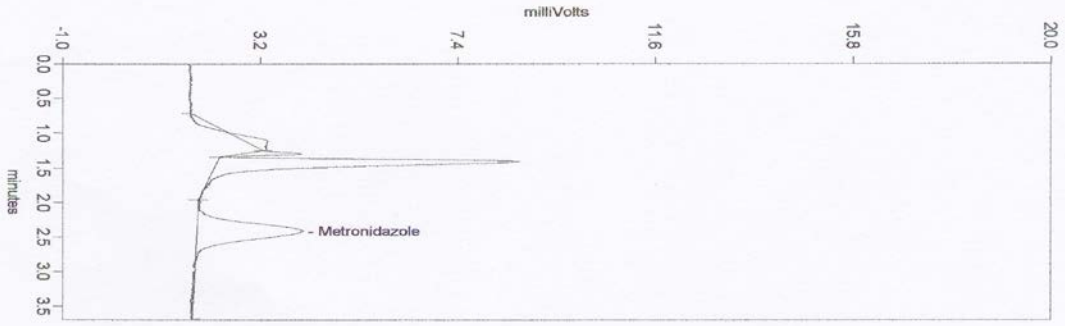
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.41	14.60	9894	0.010
TOTALS :						9894.2	

Volunteer B

```
*******************************************************************************
*                                                                             *
*  TIME :  5:09 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGAB01-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT        1.00                METHOD NAME         : METRONID
 DILUTE       :       1.00                INJ                 :  1 OF  2

 Chromatogram VGAB01-1 recorded at  5:04 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```

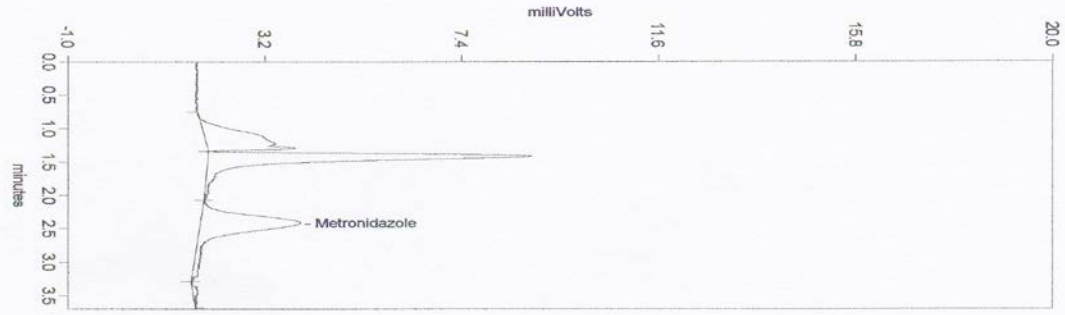
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.41	14.60	35121	0.035
TOTALS :						35121.4	

```
*******************************************************************************
*                                                                             *
*  TIME :  5:13 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGAB01-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT        1.00                METHOD NAME         : METRONID
 DILUTE       :       1.00                INJ                 :  2 OF  2

 Chromatogram VGAB01-2 recorded at  5:09 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



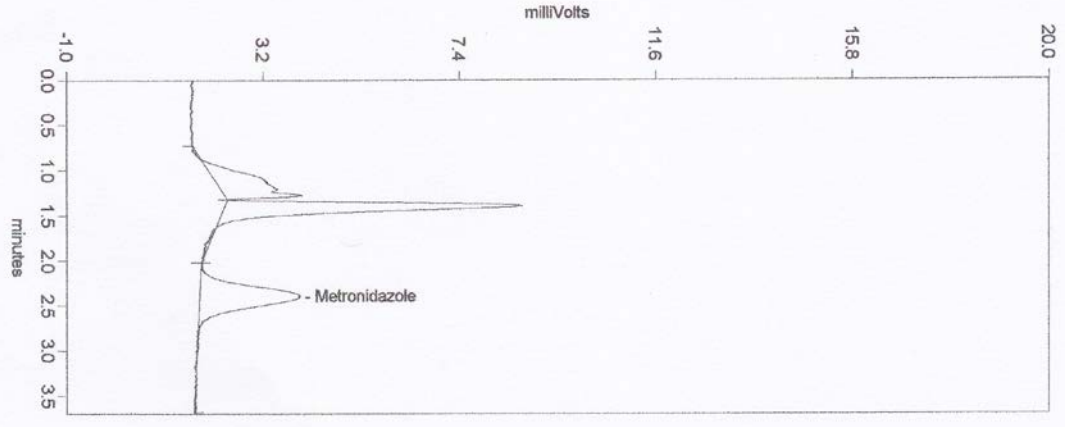
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.41	14.50	35197	0.035
TOTALS :						35197.1	

```
*******************************************************************************
*                                                                             *
*  TIME :  5:17 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006  *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGAB02-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  1 OF  2

 Chromatogram VGAB02-1 recorded at  5:13 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



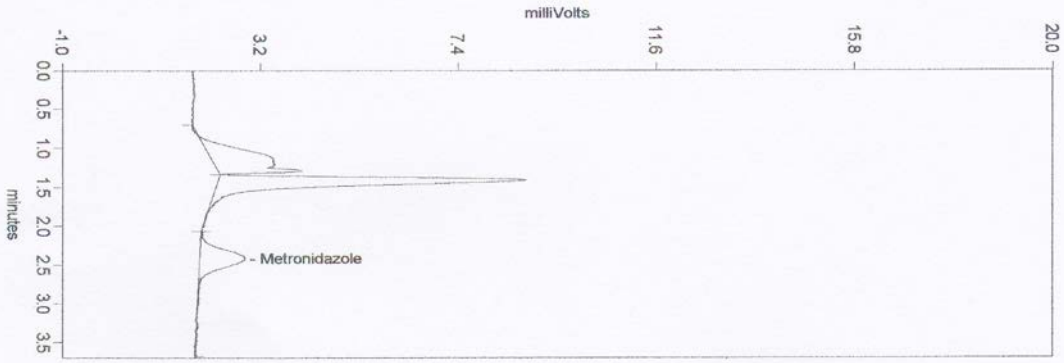
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.41	14.60	37820	0.037
TOTALS :						37820.1	

```
*******************************************************************************
*                                                                             *
*  TIME :  5:22 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006  *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGAB02-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  2 OF  2

 Chromatogram VGAB02-2 recorded at  5:17 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



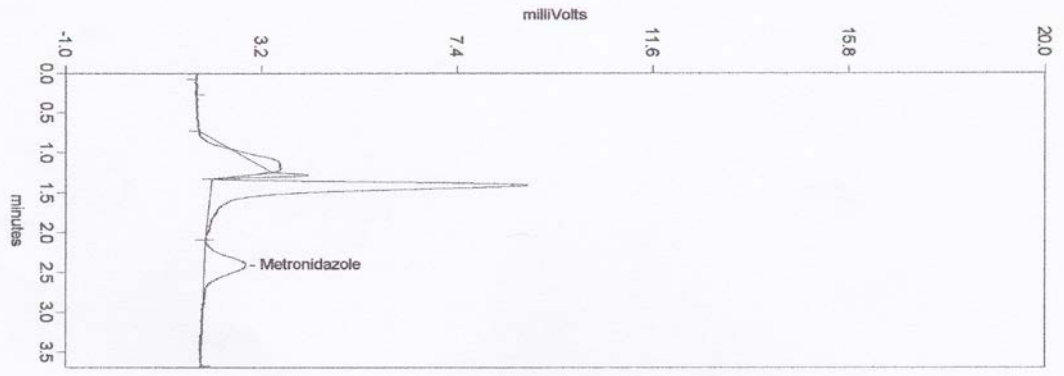
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.39	14.50	34378	0.034
TOTALS :						34378.3	

```
*******************************************************************************
*                                                                             *
*  TIME :  5:26 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGAB04-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  1 OF  2

 Chromatogram VGAB04-1 recorded at  5:22 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



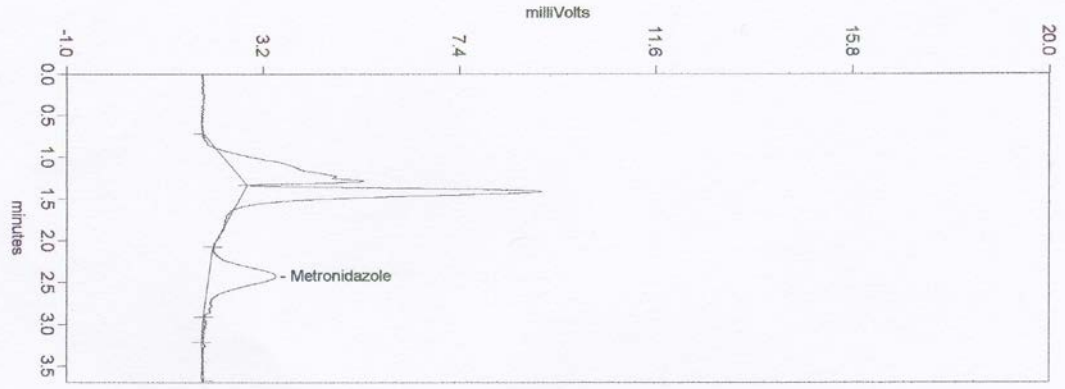
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.41	14.80	15471	0.015
TOTALS :						15471.5	

```
*******************************************************************************
*                                                                             *
*  TIME :  5:30 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGAB04-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  2 OF  2

 Chromatogram VGAB04-2 recorded at  5:26 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



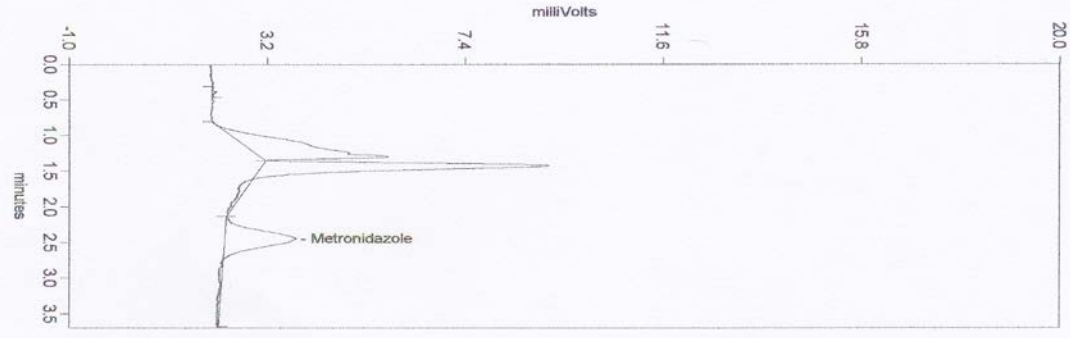
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.39	15.30	14213	0.014
TOTALS :						14212.5	

```
*******************************************************************************
*                                                                             *
*  TIME :  5:35 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                           EXTERNAL STANDARDS REPORT                         *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGAB08-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT        1.00                METHOD NAME         : METRONID
 DILUTE       :       1.00                INJ                 :  1 OF  2

 Chromatogram VGAB08-1 recorded at  5:30 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.41	15.30	24743	0.024
TOTALS :						24743.3	

```
*******************************************************************************
*                                                                             *
*  TIME :  5:39 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE : 8/12/2006 *
*                           EXTERNAL STANDARDS REPORT                         *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGAB08-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT        1.00                METHOD NAME         : METRONID
 DILUTE       :       1.00                INJ                 :  2 OF  2

 Chromatogram VGAB08-2 recorded at  5:34 pm on 08/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



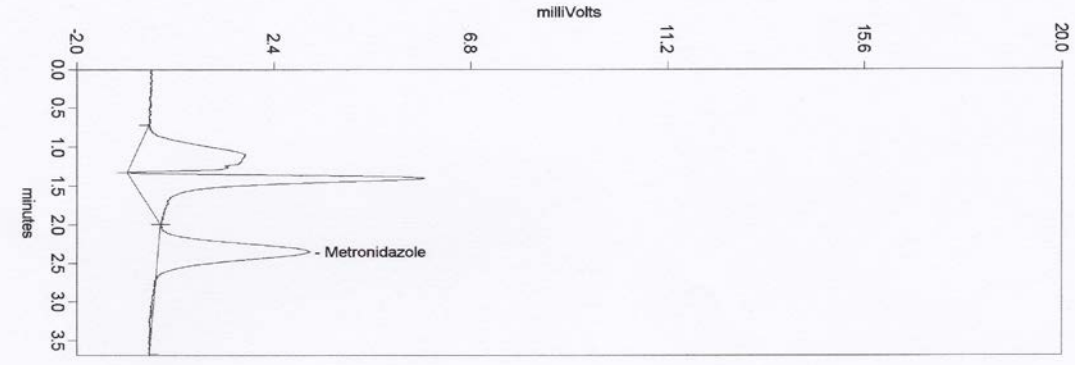
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.43	14.20	20542	0.020
TOTALS :						20541.8	

Volunteer C

```
*************************************************************************
*                                                                       *
*  TIME : 10:42 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*                                                                       *
*************************************************************************

 SAMPLE NAME :   Vaginal Tampon-1          CHROMATOGRAM FILE :    VGCC01-1
 SAMPLE TYPE :   SAMP                      CHROMATOGRAM SOURCE  : FILE
 SAMPLE AMOUNT          1.00               METHOD NAME          : METRONID
 DILUTE      :          1.00               INJ                  :  1 OF  2

 Chromatogram VGCC01-1 recorded at  9:06 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```

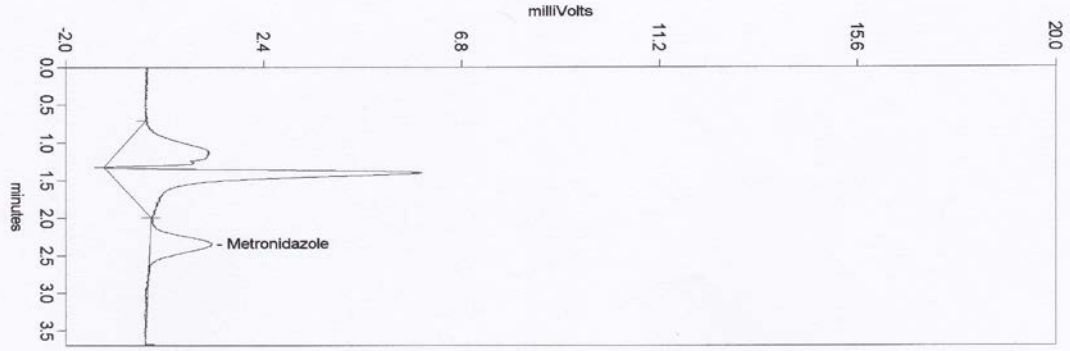
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.34	14.00	46814	0.046
TOTALS :						46813.5	

```
*************************************************************************
*                                                                       *
*  TIME : 10:42 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*                                                                       *
*************************************************************************

 SAMPLE NAME :   Vaginal Tampon-2          CHROMATOGRAM FILE :    VGCC01-2
 SAMPLE TYPE :   SAMP                      CHROMATOGRAM SOURCE  : FILE
 SAMPLE AMOUNT          1.00               METHOD NAME          : METRONID
 DILUTE      :          1.00               INJ                  :  2 OF  2

 Chromatogram VGCC01-2 recorded at  9:11 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



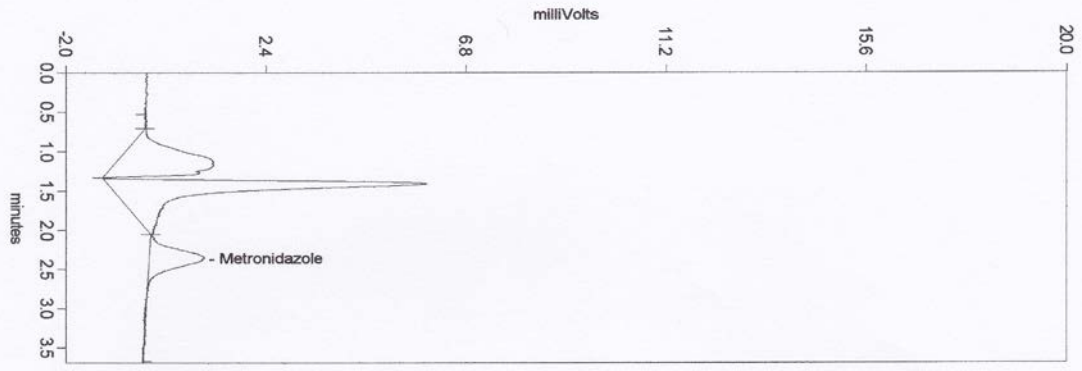
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.35	14.20	49838	0.049
TOTALS :						49838.2	

```
*************************************************************************
*                                                                       *
*  TIME : 10:42 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGCC02-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00                METHOD NAME         : METRONID
 DILUTE       :       1.00                INJ                 :  1 OF  2

 Chromatogram VGCC02-1 recorded at  8:56 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



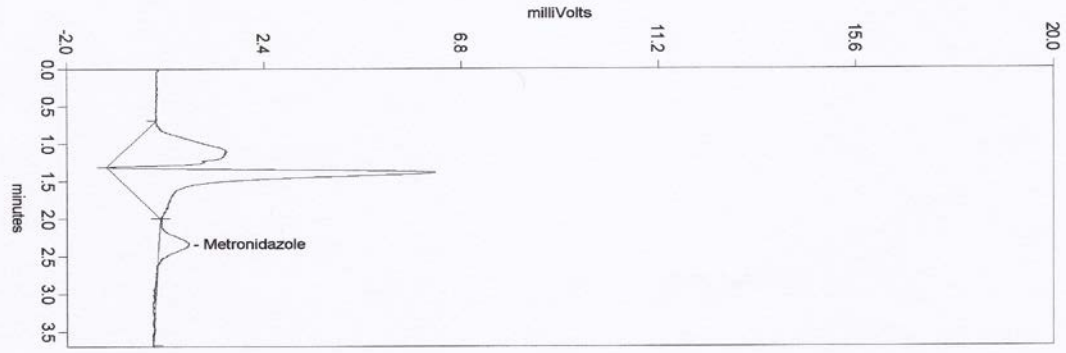
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.33	14.00	19611	0.019
TOTALS :						19611.4	

```
*************************************************************************
*                                                                       *
*  TIME : 10:42 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGCC02-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00                METHOD NAME         : METRONID
 DILUTE       :       1.00                INJ                 :  2 OF  2

 Chromatogram VGCC02-2 recorded at  9:01 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



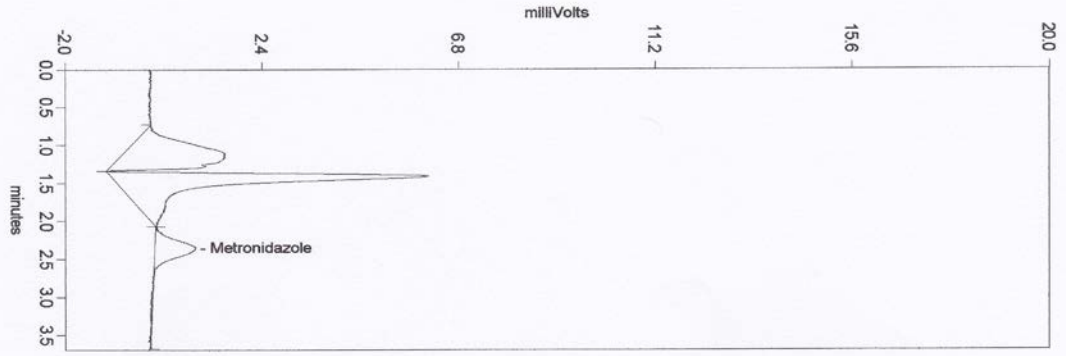
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.36	14.30	18712	0.018
TOTALS :						18711.8	

```
*******************************************************************************
*                                                                             *
*  TIME : 10:42 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGCC04-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE      :          1.00              INJ                 :  1 OF  2

 Chromatogram VGCC04-1 recorded at  8:45 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.33	13.60	8026	0.008
TOTALS :						8025.8	

```
*******************************************************************************
*                                                                             *
*  TIME : 10:42 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGCC04-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE      :          1.00              INJ                 :  2 OF  2

 Chromatogram VGCC04-2 recorded at  8:50 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



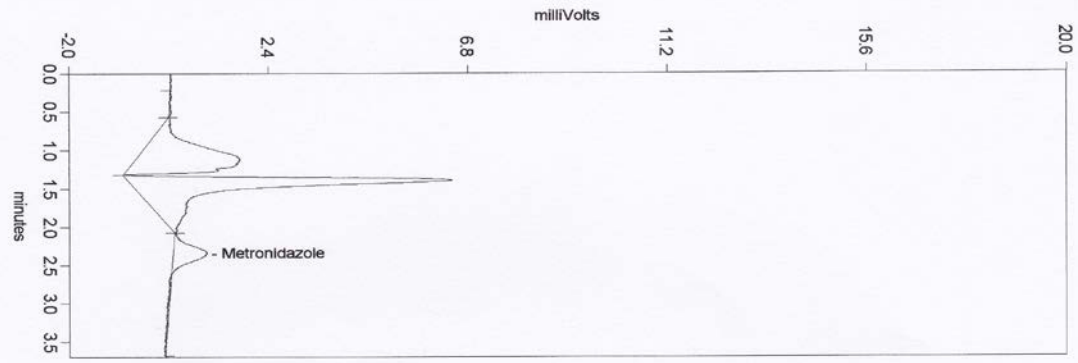
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.34	14.10	12927	0.013
TOTALS :						12926.7	

TIME : 10:42 am DELTA CHROMATOGRAPHY DATA SYSTEM DATE :11/12/2006
EXTERNAL STANDARDS REPORT

SAMPLE NAME : Vaginal Tampon-1
SAMPLE TYPE : SAMP
SAMPLE AMOUNT : 1.00
DILUTE : 1.00

CHROMATOGRAM FILE : VGCC08-1
CHROMATOGRAM SOURCE : FILE
METHOD NAME : METRONID
INJ : 1 OF 2

Chromatogram VGCC08-1 recorded at 8:35 am on 09/12/2006
Captured by analyst TLN using method : METRONID
Metronidazole content:mg/l



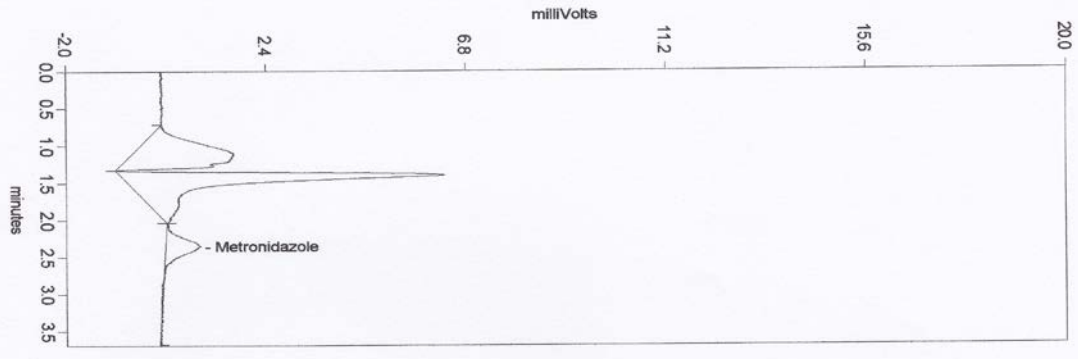
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.33	13.40	8812	0.009
TOTALS :						8812.4	

TIME : 10:42 am DELTA CHROMATOGRAPHY DATA SYSTEM DATE :11/12/2006
EXTERNAL STANDARDS REPORT

SAMPLE NAME : Vaginal Tampon-2
SAMPLE TYPE : SAMP
SAMPLE AMOUNT : 1.00
DILUTE : 1.00

CHROMATOGRAM FILE : VGCC08-2
CHROMATOGRAM SOURCE : FILE
METHOD NAME : METRONID
INJ : 2 OF 2

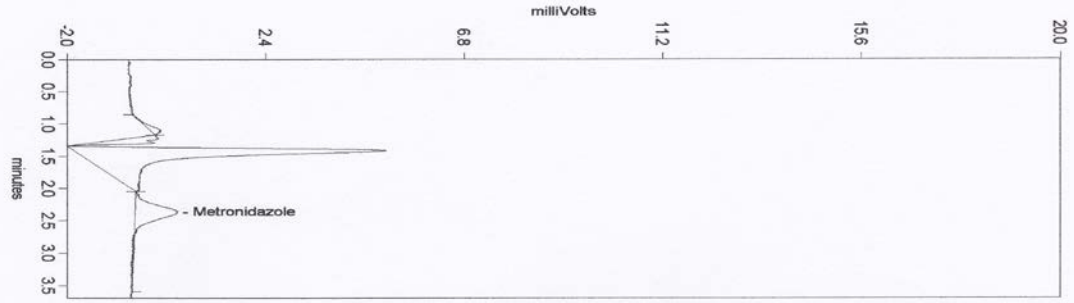
Chromatogram VGCC08-2 recorded at 8:41 am on 09/12/2006
Captured by analyst TLN using method : METRONID
Metronidazole content:mg/l



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.35	13.70	11465	0.011
TOTALS :						11465.0	

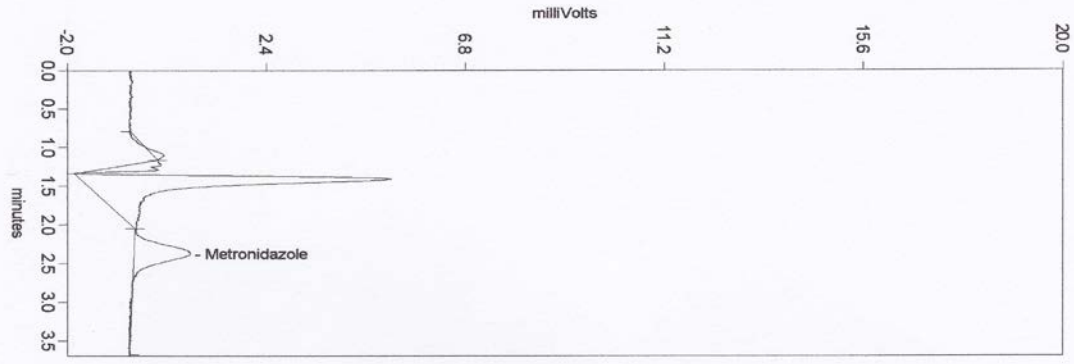
Volunteer D

```
*******************************************************************************
*                                                                             *
*  TIME : 11:03 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :   Vaginal Tampon-1          CHROMATOGRAM FILE :    VGAD01-1
 SAMPLE TYPE :   SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00               METHOD NAME           : METRONID
 DILUTE      :          1.00               INJ                   :  1 OF  2

 Chromatogram VGAD01-1 recorded at 10:09 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



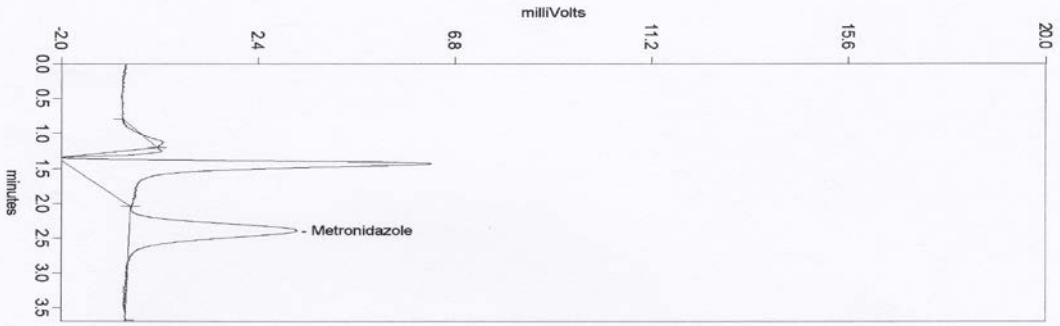
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.35	13.90	13531	0.013
TOTALS :						13531.1	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:03 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :   Vaginal Tampon-2          CHROMATOGRAM FILE :    VGAD01-2
 SAMPLE TYPE :   SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00               METHOD NAME           : METRONID
 DILUTE      :          1.00               INJ                   :  2 OF  2

 Chromatogram VGAD01-2 recorded at 10:13 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



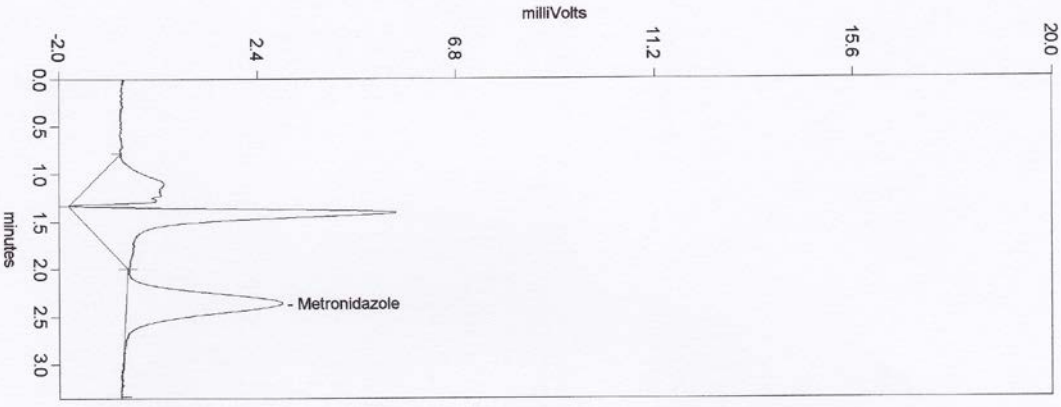
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.37	14.00	18426	0.018
TOTALS :						18425.6	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:03 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGAD02-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT         1.00               METHOD NAME         : METRONID
 DILUTE      :         1.00               INJ                 :  1 OF  2

 Chromatogram VGAD02-1 recorded at  9:59 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



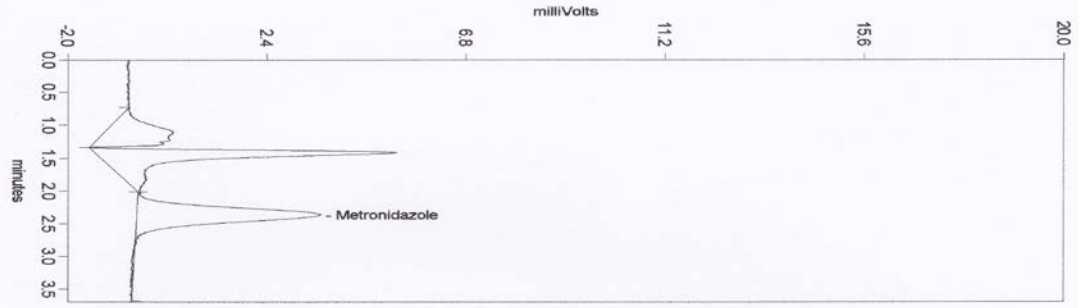
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.38	14.00	55858	0.055
TOTALS :						55858.2	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:03 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGAD02-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT         1.00               METHOD NAME         : METRONID
 DILUTE      :         1.00               INJ                 :  2 OF  2

 Chromatogram VGAD02-2 recorded at 10:05 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



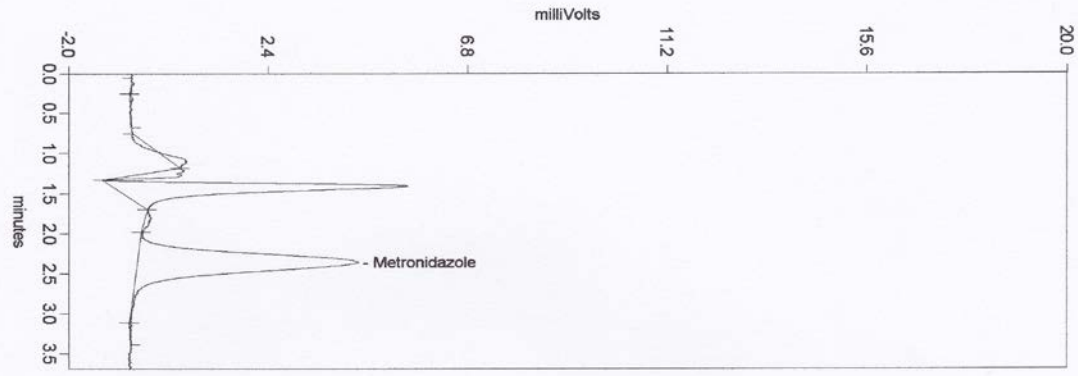
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.36	14.20	53739	0.053
TOTALS :						53739.5	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:03 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGAD04-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  1 OF  2

 Chromatogram VGAD04-1 recorded at  9:45 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



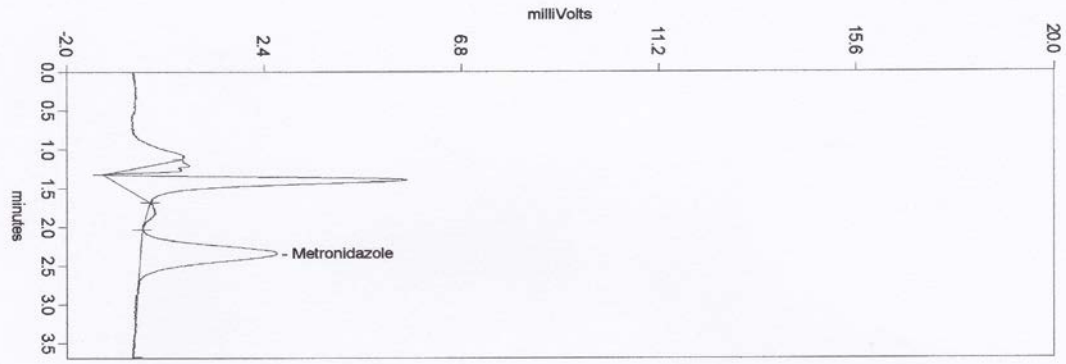
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.36	14.20	61624	0.061
TOTALS :						61624.1	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:03 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGAD04-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  2 OF  2

 Chromatogram VGAD04-2 recorded at  9:50 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



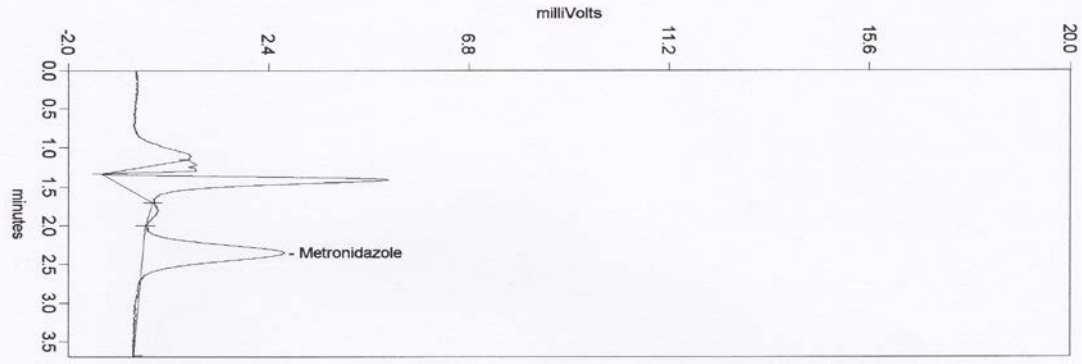
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
7	1		Metronidazol	2.36	14.30	76743	0.075
TOTALS :						76742.6	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:03 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                           EXTERNAL STANDARDS REPORT                         *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGAD08-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  1 OF  2

 Chromatogram VGAD08-1 recorded at  9:36 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.33	14.10	46527	0.046
TOTALS :						46527.3	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:03 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                           EXTERNAL STANDARDS REPORT                         *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGAD08-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  2 OF  2

 Chromatogram VGAD08-2 recorded at  9:40 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



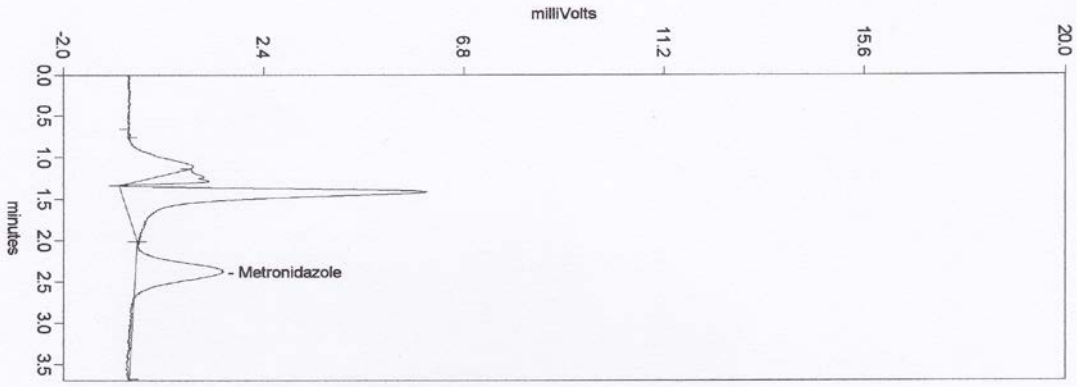
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.34	14.20	45530	0.045
TOTALS :						45530.2	

Volunteer E

```
*******************************************************************************
*                                                                             *
*  TIME : 11:19 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :   Vaginal Tampon-1          CHROMATOGRAM FILE :    VGAE01-1
 SAMPLE TYPE :   SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00               METHOD NAME         : METRONID
 DILUTE       :         1.00               INJ                 :  1 OF  2

 Chromatogram VGAE01-1 recorded at 11:17 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```

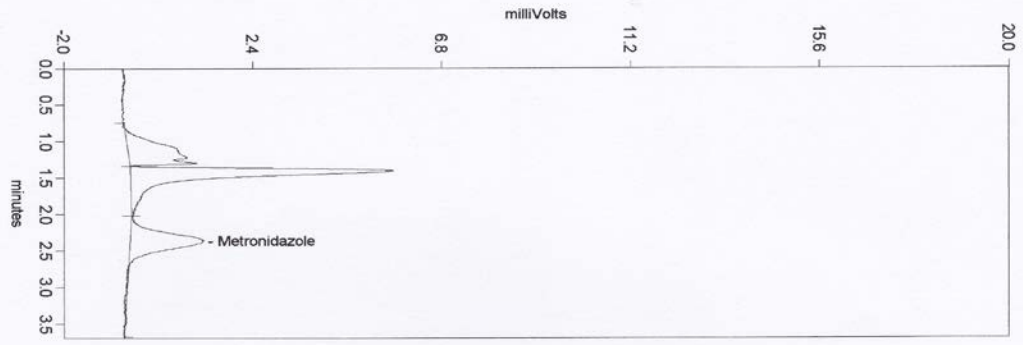
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.37	14.50	30341	0.030
TOTALS :						30341.1	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:19 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :   Vaginal Tampon-2          CHROMATOGRAM FILE :    VGAE01-2
 SAMPLE TYPE :   SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00               METHOD NAME         : METRONID
 DILUTE       :         1.00               INJ                 :  2 OF  2

 Chromatogram VGAE01-2 recorded at 11:23 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



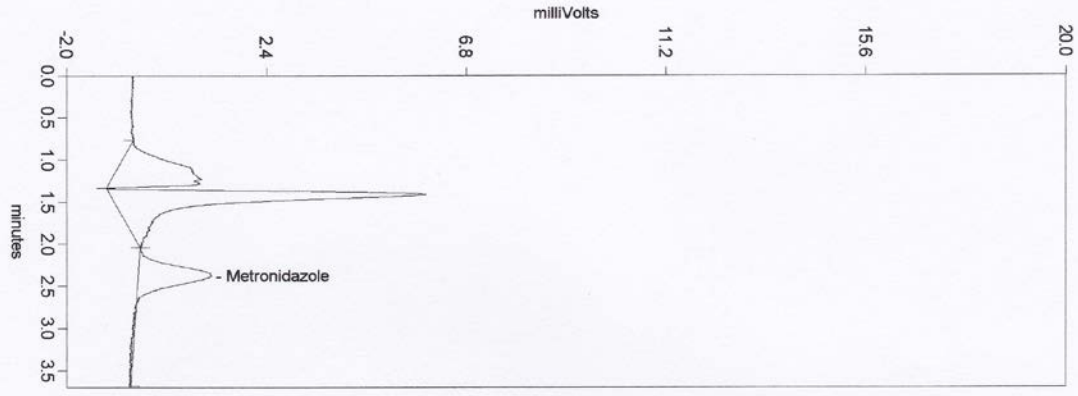
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.38	14.20	26603	0.026
TOTALS :						26602.8	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:18 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*                                                                             *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGAE02-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00                METHOD NAME         : METRONID
 DILUTE       :       1.00                INJ                 :  1 OF  2

 Chromatogram VGAE02-1 recorded at 11:07 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



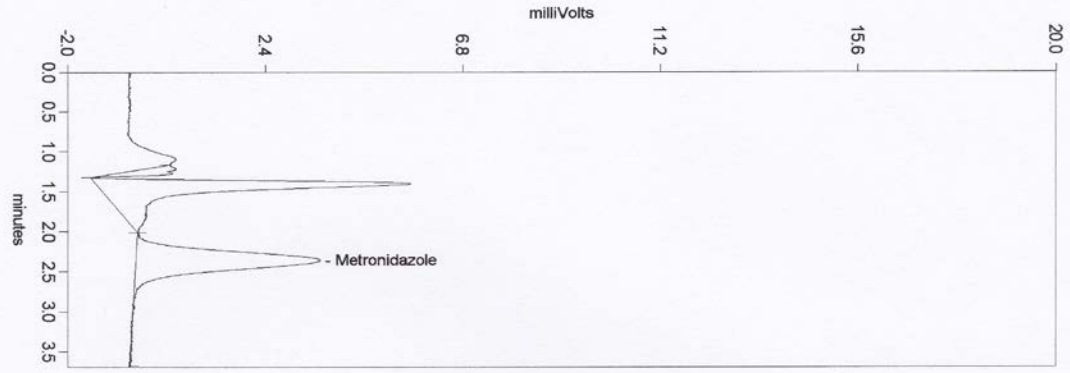
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.36	13.80	25604	0.025
TOTALS :						25604.4	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:19 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*                                                                             *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGAE02-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00                METHOD NAME         : METRONID
 DILUTE       :       1.00                INJ                 :  2 OF  2

 Chromatogram VGAE02-2 recorded at 11:13 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.37	14.30	22581	0.022
TOTALS :						22580.9	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:18 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGAE04-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00                METHOD NAME         : METRONID
 DILUTE       :       1.00                INJ                 :  1 OF  2

 Chromatogram VGAE04-1 recorded at 10:58 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.35	14.30	64668	0.064
TOTALS :						64668.1	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:18 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
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 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGAE04-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00                METHOD NAME         : METRONID
 DILUTE       :       1.00                INJ                 :  2 OF  2

 Chromatogram VGAE04-2 recorded at 11:03 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```

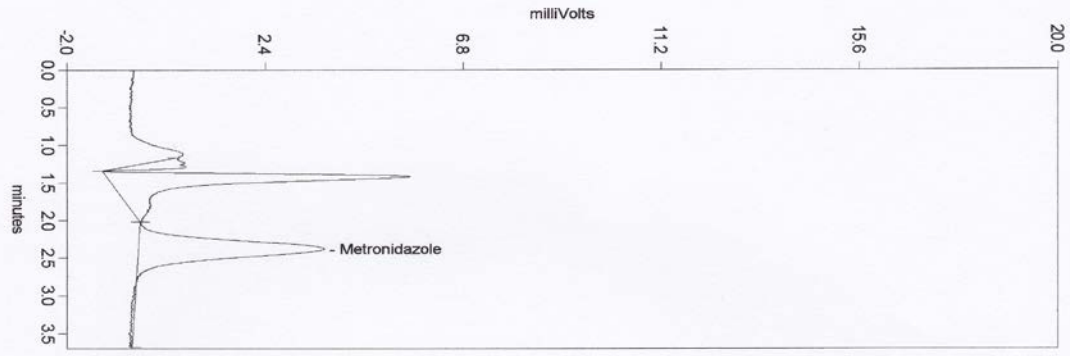

PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
3	1		Metronidazol	2.38	14.30	61986	0.061
TOTALS :						61986.0	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:18 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                           EXTERNAL STANDARDS REPORT                         *
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 SAMPLE NAME :  Vaginal Tampon-1          CHROMATOGRAM FILE :   VGAE08-1
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00                METHOD NAME         : METRONID
 DILUTE       :       1.00                INJ                 :  1 OF  2

 Chromatogram VGAE08-1 recorded at 10:46 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```
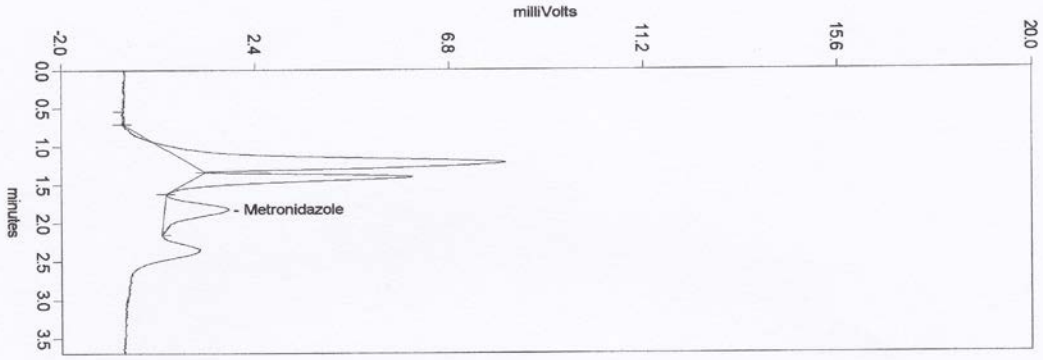
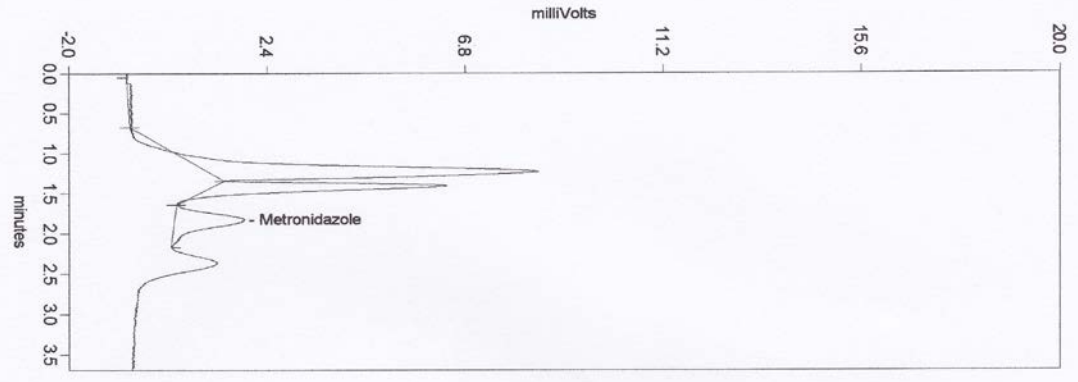



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	1.82	11.20	17811	0.018
TOTALS :						17810.9	

```
*******************************************************************************
*                                                                             *
*  TIME : 11:18 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                           EXTERNAL STANDARDS REPORT                         *
*******************************************************************************

 SAMPLE NAME :  Vaginal Tampon-2          CHROMATOGRAM FILE :   VGAE08-2
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00                METHOD NAME         : METRONID
 DILUTE       :       1.00                INJ                 :  2 OF  2

 Chromatogram VGAE08-2 recorded at 10:52 am on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```

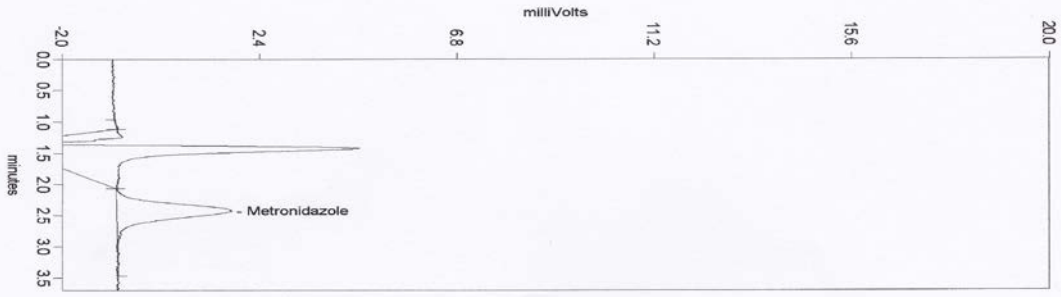


PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	1.82	11.10	19083	0.019
TOTALS :						19082.7	

2. Serum

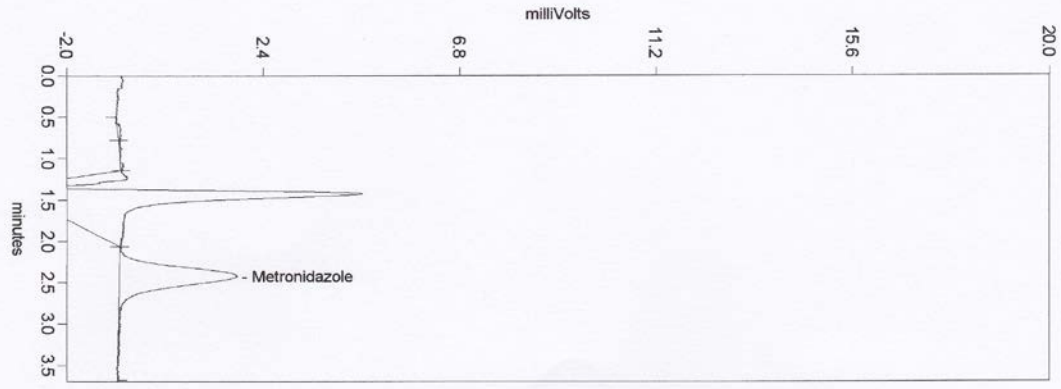
Volunteer A

```
*************************************************************************
*                                                                       *
*  TIME :  9:11 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*************************************************************************

 SAMPLE NAME :   SERUM-1                  CHROMATOGRAM FILE :    SERUA1-1
 SAMPLE TYPE :   SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE      :          1.00              INJ                 :  1 OF  2

 Chromatogram SERUA1-1 recorded at  1:47 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



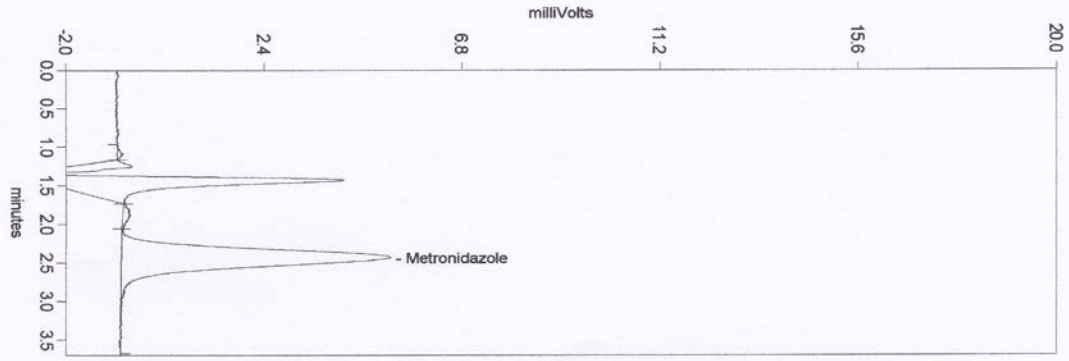
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.42	14.20	40954	0.040
TOTALS :						40954.0	

```
*************************************************************************
*                                                                       *
*  TIME :  9:11 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*************************************************************************

 SAMPLE NAME :   SERUM-2                  CHROMATOGRAM FILE :    SERUA1-2
 SAMPLE TYPE :   SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE      :          1.00              INJ                 :  2 OF  2

 Chromatogram SERUA1-2 recorded at  1:52 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



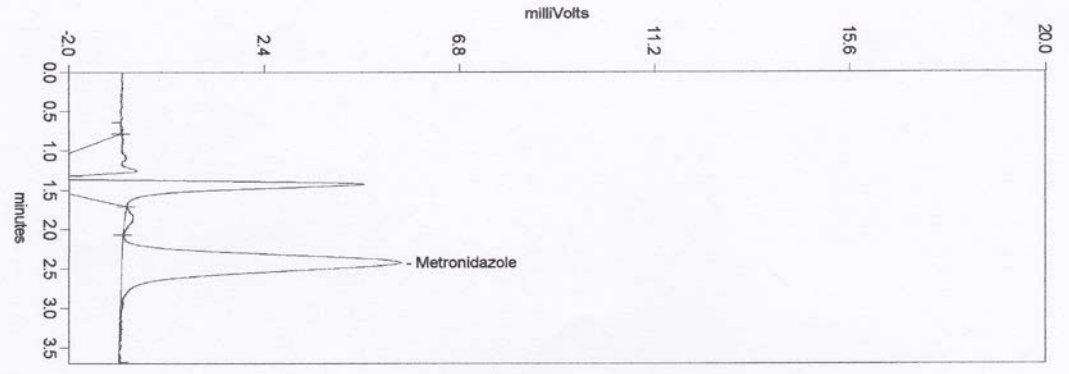
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.42	14.30	42217	0.042
TOTALS :						42216.7	

```
*******************************************************************************
*                                                                             *
*  TIME :  9:11 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :  SERUM-1                  CHROMATOGRAM FILE :   SERUA2-1
 SAMPLE TYPE :  SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00               METHOD NAME         : METRONID
 DILUTE       :       1.00               INJ                 :  1 OF  2

 Chromatogram SERUA2-1 recorded at  1:35 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.43	14.20	93014	0.091
TOTALS :						93013.6	

```
*******************************************************************************
*                                                                             *
*  TIME :  9:11 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :  SERUM-2                  CHROMATOGRAM FILE :   SERUA2-2
 SAMPLE TYPE :  SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00               METHOD NAME         : METRONID
 DILUTE       :       1.00               INJ                 :  2 OF  2

 Chromatogram SERUA2-2 recorded at  1:42 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



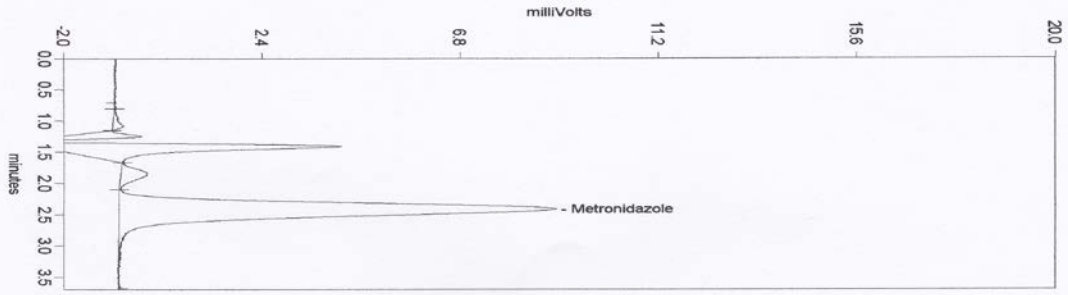
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.42	13.90	97300	0.096
TOTALS :						97299.8	

TIME : 9:11 am DELTA CHROMATOGRAPHY DATA SYSTEM DATE :11/12/2006
EXTERNAL STANDARDS REPORT

SAMPLE NAME : SERUM-1
SAMPLE TYPE : SAMP
SAMPLE AMOUNT 1.00
DILUTE : 1.00

CHROMATOGRAM FILE : SERUA4-1
CHROMATOGRAM SOURCE : FILE
METHOD NAME : METRONID
INJ : 1 OF 2

Chromatogram SERUA4-1 recorded at 1:20 pm on 09/12/2006
Captured by analyst TLN using method : METRONID
Metronidazole content:mg/l



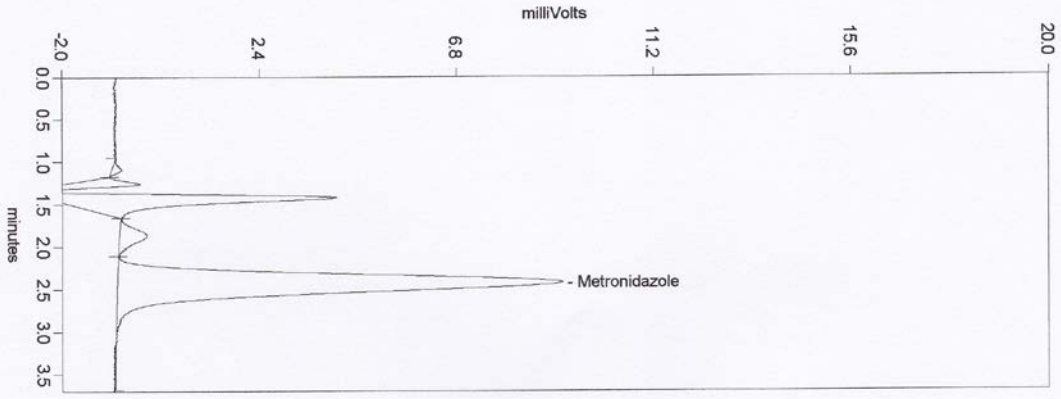
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
6	1		Metronidazol	2.41	13.60	145222	0.143
TOTALS :						145222.1	

TIME : 9:11 am DELTA CHROMATOGRAPHY DATA SYSTEM DATE :11/12/2006
EXTERNAL STANDARDS REPORT

SAMPLE NAME : SERUM-2
SAMPLE TYPE : SAMP
SAMPLE AMOUNT 1.00
DILUTE : 1.00

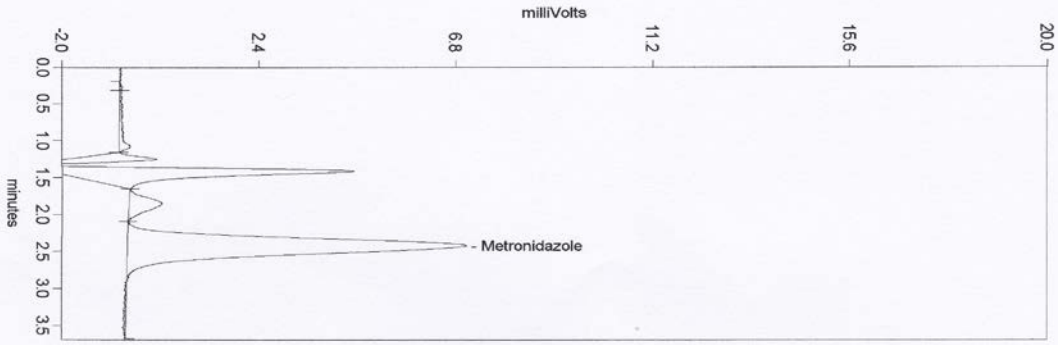
CHROMATOGRAM FILE : SERUA4-2
CHROMATOGRAM SOURCE : FILE
METHOD NAME : METRONID
INJ : 2 OF 2

Chromatogram SERUA4-2 recorded at 1:29 pm on 09/12/2006
Captured by analyst TLN using method : METRONID
Metronidazole content:mg/l



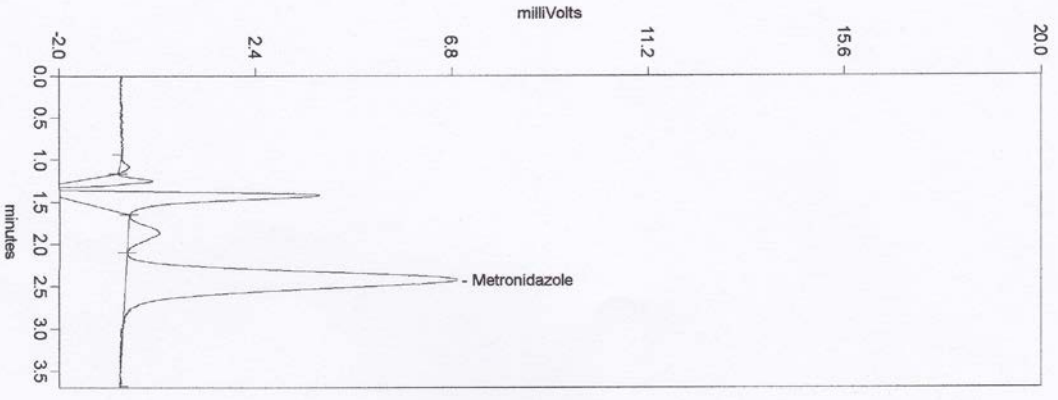
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.42	13.50	146396	0.144
TOTALS :						146396.3	

```
*******************************************************************************
*                                                                             *
*  TIME :  9:11 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :  SERUM-1                    CHROMATOGRAM FILE :   SERUA8-1
 SAMPLE TYPE :  SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00                 METHOD NAME         : METRONID
 DILUTE       :       1.00                 INJ                 :  1 OF  2

 Chromatogram SERUA8-1 recorded at 12:50 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
6	1		Metronidazol	2.42	13.70	111208	0.109
TOTALS :						111208.2	

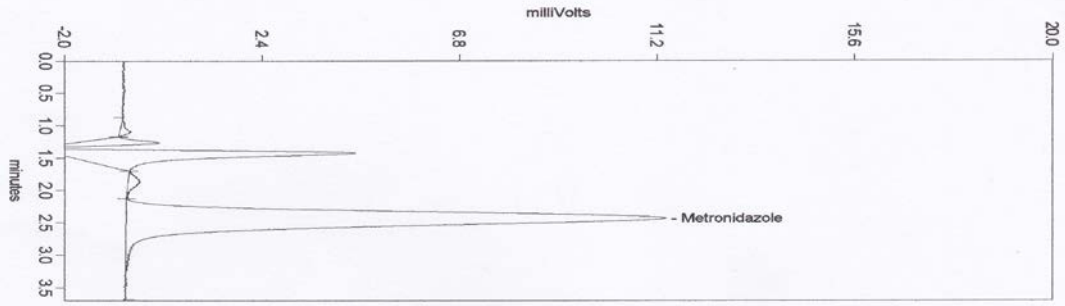
```
*******************************************************************************
*                                                                             *
*  TIME :  9:11 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :  SERUM-2                    CHROMATOGRAM FILE :   SERUA8-2
 SAMPLE TYPE :  SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00                 METHOD NAME         : METRONID
 DILUTE       :       1.00                 INJ                 :  2 OF  2

 Chromatogram SERUA8-2 recorded at  1:00 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.42	13.70	110966	0.109
TOTALS :						110965.7	

Volunteer B

```
*************************************************************************
*                                                                       *
*  TIME :  9:24 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                        EXTERNAL STANDARDS REPORT                      *
*************************************************************************

 SAMPLE NAME :   SERUM-1                  CHROMATOGRAM FILE :    SERUB1-1
 SAMPLE TYPE :   SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  1 OF  2

 Chromatogram SERUB1-1 recorded at  2:53 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.43	13.70	181434	0.178
TOTALS :						181434.2	

```
*************************************************************************
*                                                                       *
*  TIME :  9:24 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                        EXTERNAL STANDARDS REPORT                      *
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 SAMPLE NAME :   SERUM-2                  CHROMATOGRAM FILE :    SERUB1-2
 SAMPLE TYPE :   SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE       :         1.00              INJ                 :  2 OF  2

 Chromatogram SERUB1-2 recorded at  3:01 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```

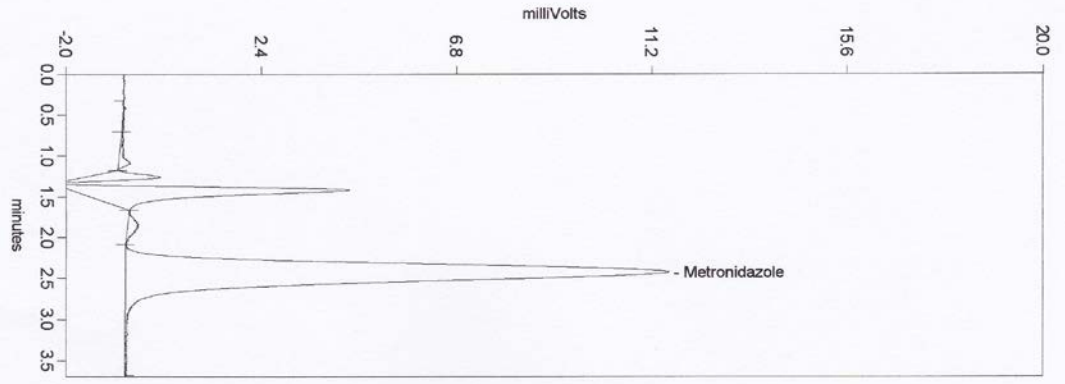

PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
6	1		Metronidazol	2.42	13.60	184427	0.181
TOTALS :						184426.9	

```
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*  TIME :  9:24 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                              EXTERNAL STANDARDS REPORT                      *
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 SAMPLE NAME :   SERUM-1                    CHROMATOGRAM FILE :    SERUB2-1
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE  : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME          : METRONID
 DILUTE      :          1.00                INJ                  :  1 OF  2

 Chromatogram SERUB2-1 recorded at  2:42 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```

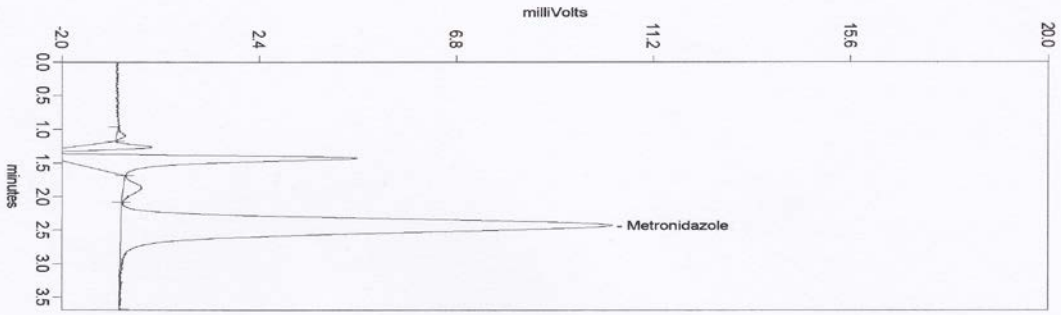

PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.43	13.70	165071	0.162
TOTALS :						165070.9	

```
*******************************************************************************
*                                                                             *
*  TIME :  9:24 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                              EXTERNAL STANDARDS REPORT                      *
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 SAMPLE NAME :   SERUM-2                    CHROMATOGRAM FILE :    SERUB2-2
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE  : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME          : METRONID
 DILUTE      :          1.00                INJ                  :  2 OF  2

 Chromatogram SERUB2-2 recorded at  2:48 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```

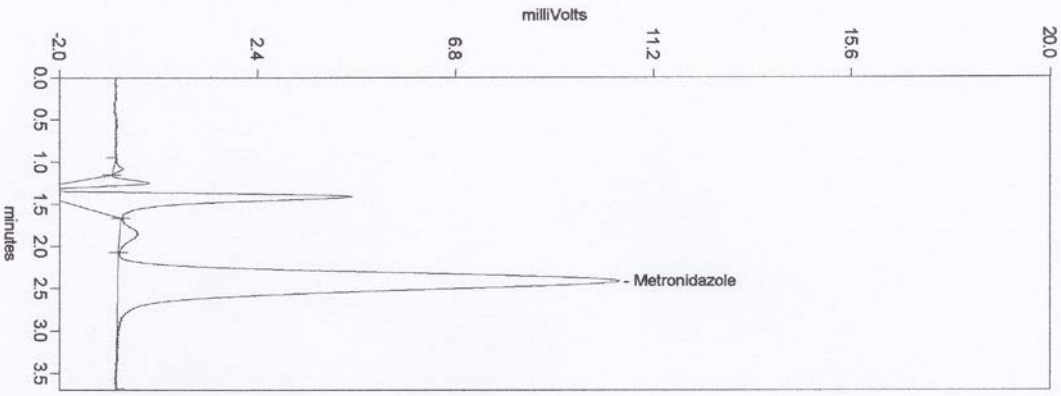

PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.41	13.60	166829	0.164
TOTALS :						166829.0	

```
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*  TIME :  9:24 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*************************************************************************

 SAMPLE NAME :   SERUM-1                    CHROMATOGRAM FILE :    SERUB4-1
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME           : METRONID
 DILUTE      :          1.00                INJ                   :  1 OF  2

 Chromatogram SERUB4-1 recorded at  2:29 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```
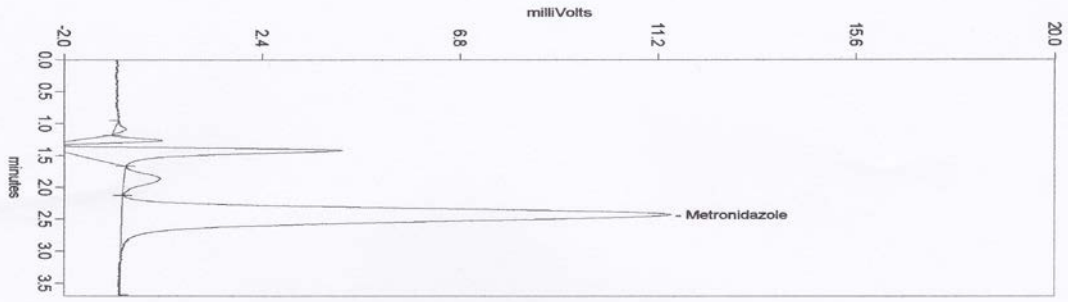
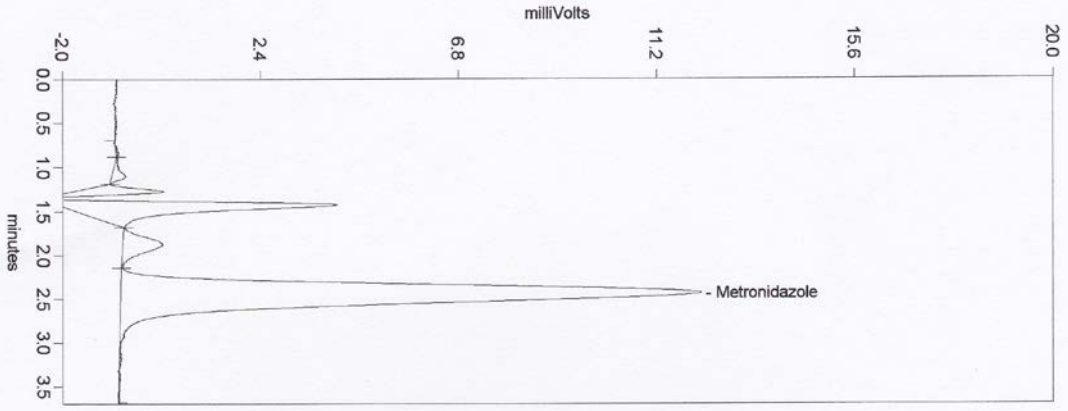



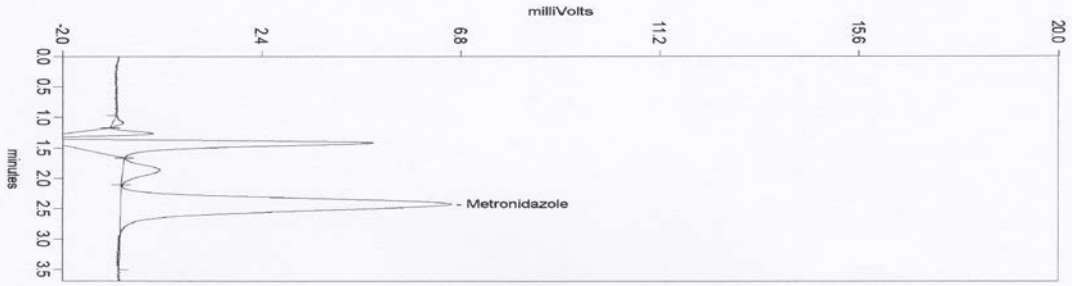
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.43	13.40	178836	0.176
TOTALS :						178835.6	

```
*************************************************************************
*                                                                       *
*  TIME :  9:24 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*************************************************************************

 SAMPLE NAME :   SERUM-2                    CHROMATOGRAM FILE :    SERUB4-2
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME           : METRONID
 DILUTE      :          1.00                INJ                   :  2 OF  2

 Chromatogram SERUB4-2 recorded at  2:35 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



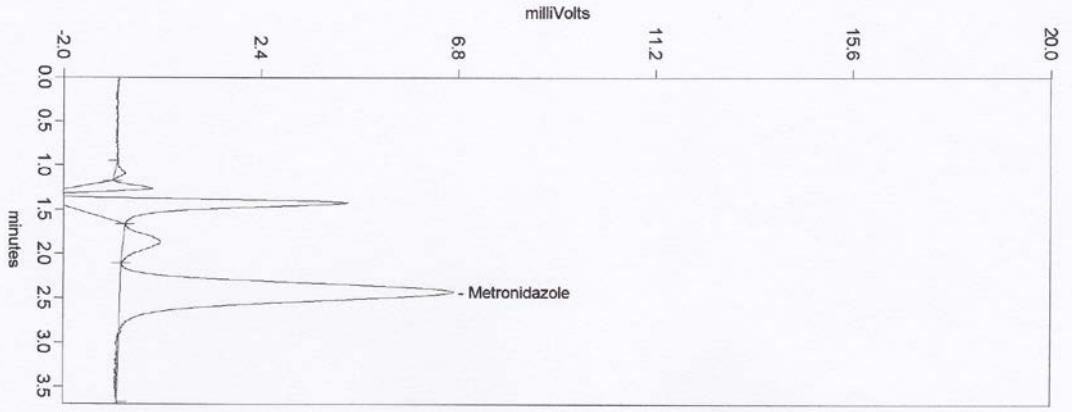
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
6	1		Metronidazol	2.43	13.40	188979	0.186
TOTALS :						188978.8	

```
*******************************************************************************
*                                                                             *
*  TIME :  9:24 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :   SERUM-1                    CHROMATOGRAM FILE :    SERUB8-1
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME           : METRONID
 DILUTE      :          1.00                INJ                   :  1 OF  2

 Chromatogram SERUB8-1 recorded at  2:16 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.42	13.50	106763	0.105
TOTALS :						106762.9	

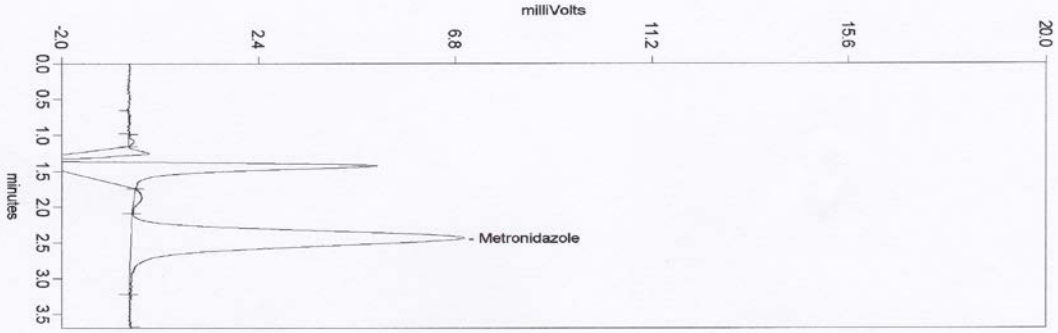
```
*******************************************************************************
*                                                                             *
*  TIME :  9:24 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :   SERUM-2                    CHROMATOGRAM FILE :    SERUB8-2
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME           : METRONID
 DILUTE      :          1.00                INJ                   :  2 OF  2

 Chromatogram SERUB8-2 recorded at  2:23 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.42	13.50	107614	0.106
TOTALS :						107613.7	

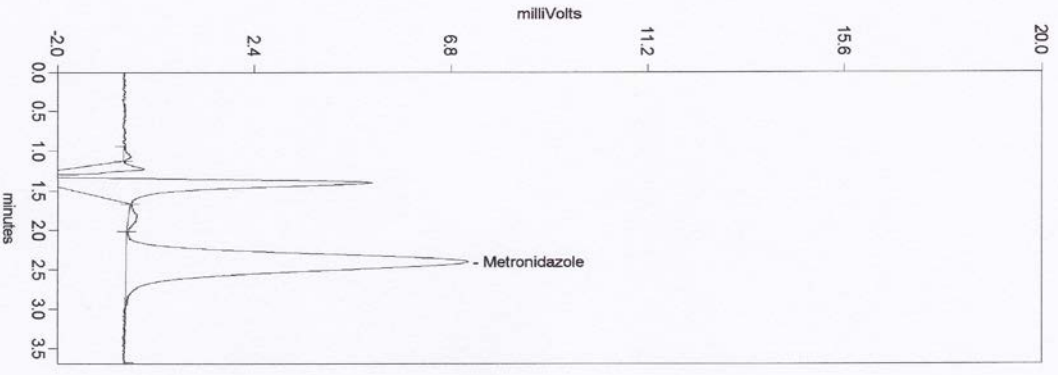
Volunteer C

```
*******************************************************************************
*                                                                             *
*  TIME :  9:47 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :   SERUM-1                    CHROMATOGRAM FILE :    SERUC1-1
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME         : METRONID
 DILUTE      :          1.00                INJ                 :  1 OF  2

 Chromatogram SERUC1-1 recorded at  3:54 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



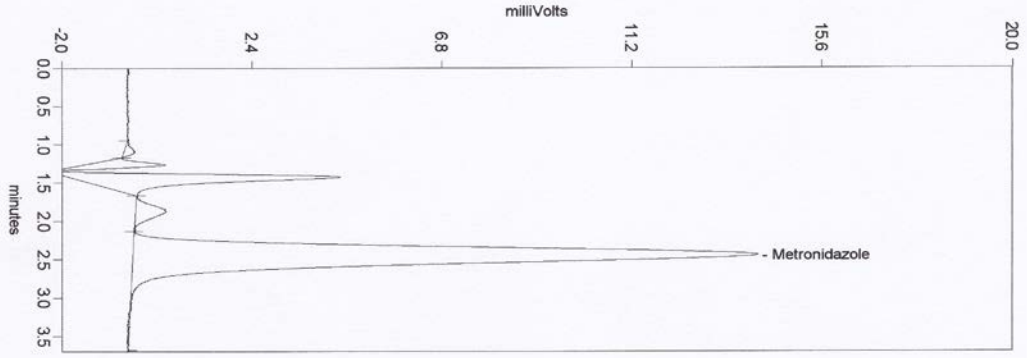
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
6	1		Metronidazol	2.43	14.20	117463	0.115
TOTALS :						117463.0	

```
*******************************************************************************
*                                                                             *
*  TIME :  9:47 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                          *
*******************************************************************************

 SAMPLE NAME :   SERUM-2                    CHROMATOGRAM FILE :    SERUC1-2
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME         : METRONID
 DILUTE      :          1.00                INJ                 :  2 OF  2

 Chromatogram SERUC1-2 recorded at  4:00 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



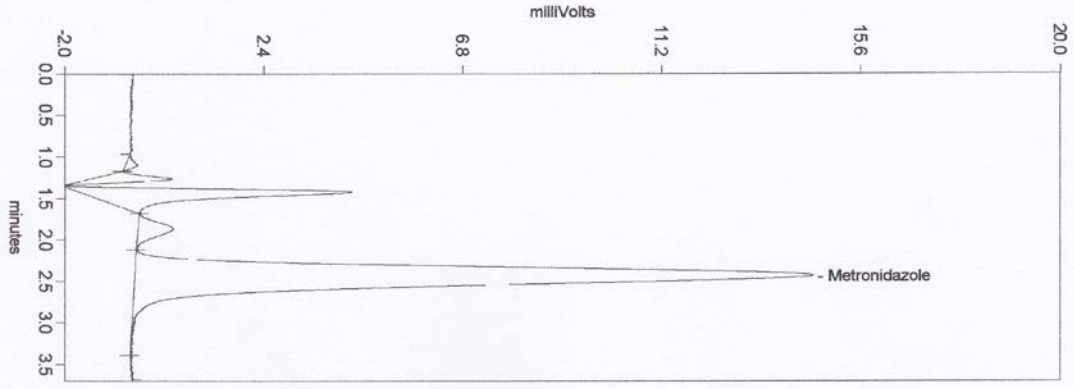
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.40	14.10	118520	0.117
TOTALS :						118520.5	

```
*******************************************************************************
*                                                                             *
*  TIME :  9:47 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :  SERUM-1                  CHROMATOGRAM FILE :   SERUC2-1
 SAMPLE TYPE :  SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00               METHOD NAME         : METRONID
 DILUTE       :       1.00               INJ                 :  1 OF  2

 Chromatogram SERUC2-1 recorded at  3:43 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



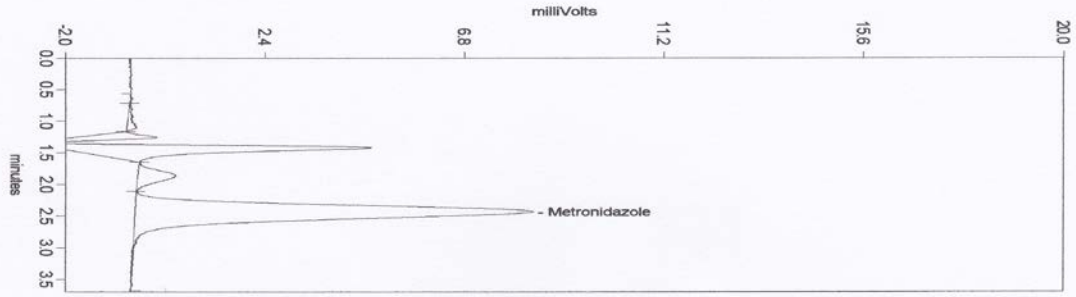
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.44	13.70	215560	0.212
TOTALS :						215559.8	

```
*******************************************************************************
*                                                                             *
*  TIME :  9:47 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :  SERUM-2                  CHROMATOGRAM FILE :   SERUC2-2
 SAMPLE TYPE :  SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00               METHOD NAME         : METRONID
 DILUTE       :       1.00               INJ                 :  2 OF  2

 Chromatogram SERUC2-2 recorded at  3:50 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



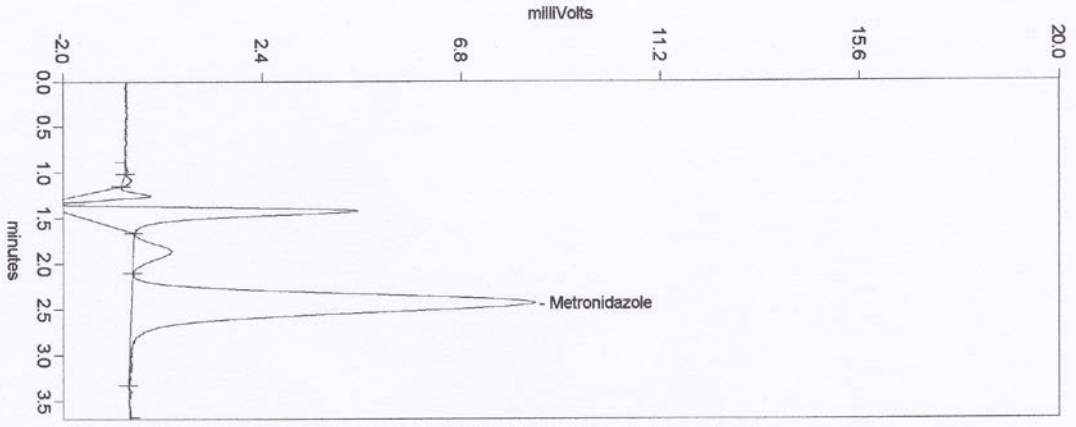
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.43	13.60	224803	0.221
TOTALS :						224803.4	

```
*************************************************************************
*                                                                       *
*  TIME :  9:47 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*************************************************************************

 SAMPLE NAME :   SERUM-1                  CHROMATOGRAM FILE :    SERUC4-1
 SAMPLE TYPE :   SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE      :          1.00              INJ                 :  1 OF  2

 Chromatogram SERUC4-1 recorded at  3:29 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



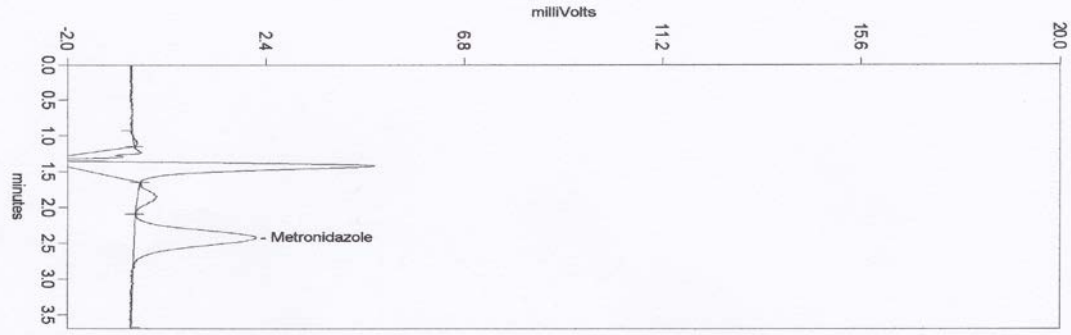
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
6	1		Metronidazol	2.43	13.80	132741	0.131
TOTALS :						132740.7	

```
*************************************************************************
*                                                                       *
*  TIME :  9:47 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*************************************************************************

 SAMPLE NAME :   SERUM-2                  CHROMATOGRAM FILE :    SERUC4-2
 SAMPLE TYPE :   SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE      :          1.00              INJ                 :  2 OF  2

 Chromatogram SERUC4-2 recorded at  3:38 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



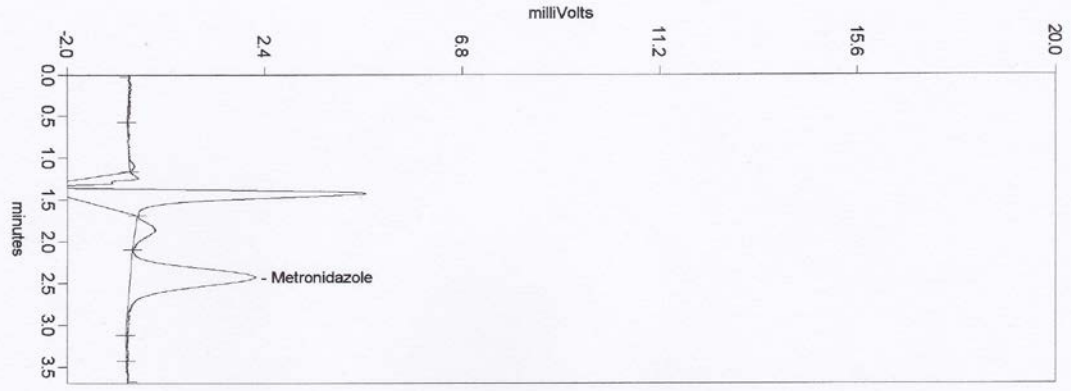
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
6	1		Metronidazol	2.43	13.80	134930	0.133
TOTALS :						134930.0	

```
*******************************************************************************
*                                                                             *
*  TIME :  9:46 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   SERUM-1                    CHROMATOGRAM FILE :    SERUC8-1
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME          : METRONID
 DILUTE      :          1.00                INJ                  :  1 OF  2

 Chromatogram SERUC8-1 recorded at  3:18 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.41	14.30	41602	0.041
TOTALS :						41602.0	

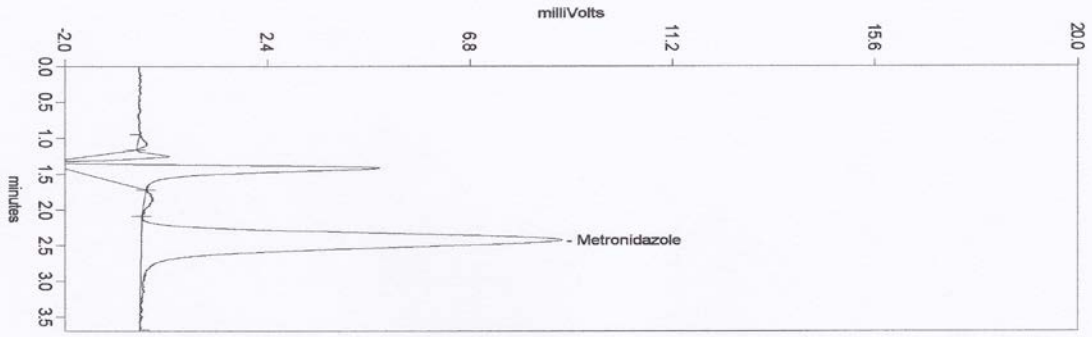
```
*******************************************************************************
*                                                                             *
*  TIME :  9:46 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   SERUM-2                    CHROMATOGRAM FILE :    SERUC8-2
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME          : METRONID
 DILUTE      :          1.00                INJ                  :  2 OF  2

 Chromatogram SERUC8-2 recorded at  3:23 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
6	1		Metronidazol	2.42	14.20	44193	0.043
TOTALS :						44193.0	

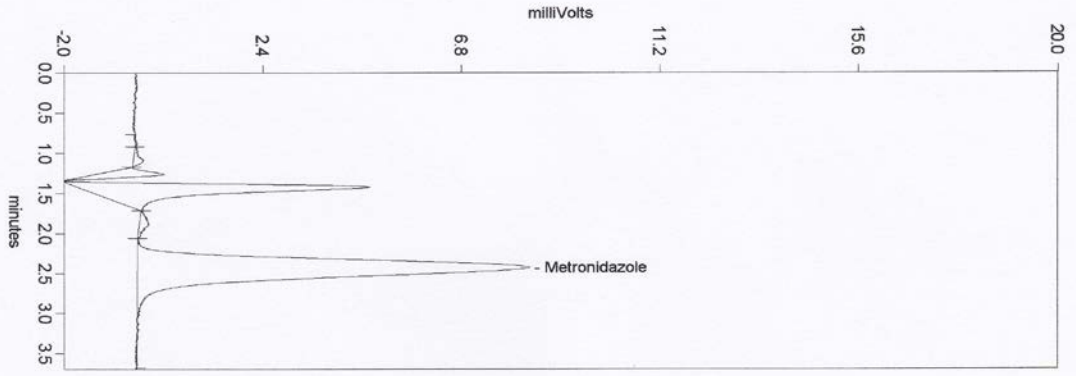
Volunteer D

```
*******************************************************************************
*                                                                             *
*  TIME : 10:04 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   SERUM-1                    CHROMATOGRAM FILE :    SERUD1-1
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME         : METRONID
 DILUTE      :          1.00                INJ                 :  1 OF  2

 Chromatogram SERUD1-1 recorded at  4:59 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



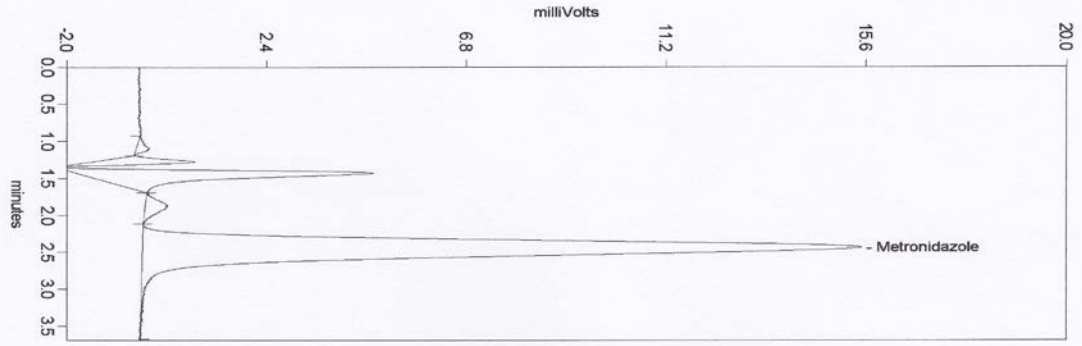
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.42	13.80	139138	0.137
TOTALS :						139138.0	

```
*******************************************************************************
*                                                                             *
*  TIME : 10:04 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   SERUM-2                    CHROMATOGRAM FILE :    SERUD1-2
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME         : METRONID
 DILUTE      :          1.00                INJ                 :  2 OF  2

 Chromatogram SERUD1-2 recorded at  5:03 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



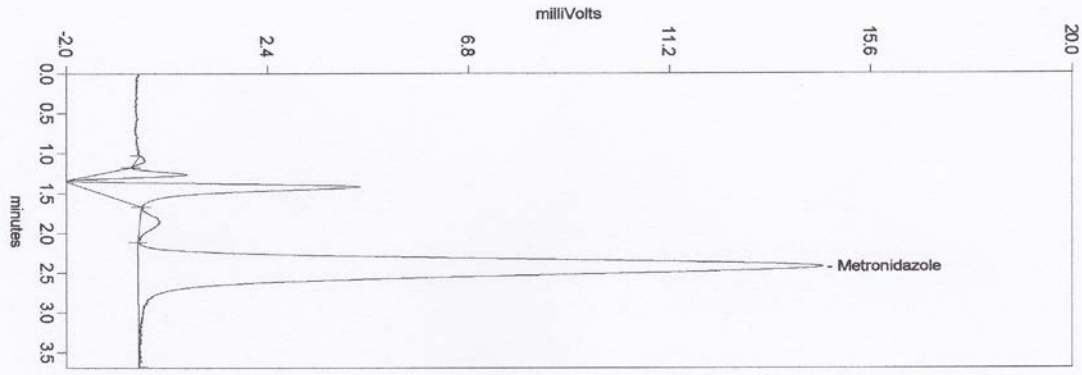
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
6	1		Metronidazol	2.42	13.80	131622	0.129
TOTALS :						131621.7	

```
*************************************************************************
*                                                                       *
*  TIME : 10:04 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*************************************************************************

 SAMPLE NAME :  SERUM-1                  CHROMATOGRAM FILE :   SERUD2-1
 SAMPLE TYPE :  SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00               METHOD NAME         : METRONID
 DILUTE       :       1.00               INJ                 :  1 OF  2

 Chromatogram SERUD2-1 recorded at  4:44 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



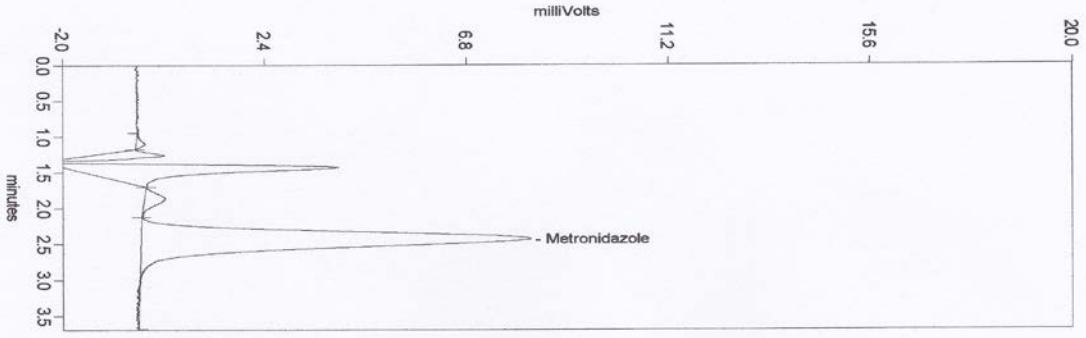
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.43	13.40	233206	0.229
TOTALS :						233206.4	

```
*************************************************************************
*                                                                       *
*  TIME : 10:04 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                          EXTERNAL STANDARDS REPORT                    *
*************************************************************************

 SAMPLE NAME :  SERUM-2                  CHROMATOGRAM FILE :   SERUD2-2
 SAMPLE TYPE :  SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00               METHOD NAME         : METRONID
 DILUTE       :       1.00               INJ                 :  2 OF  2

 Chromatogram SERUD2-2 recorded at  4:53 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



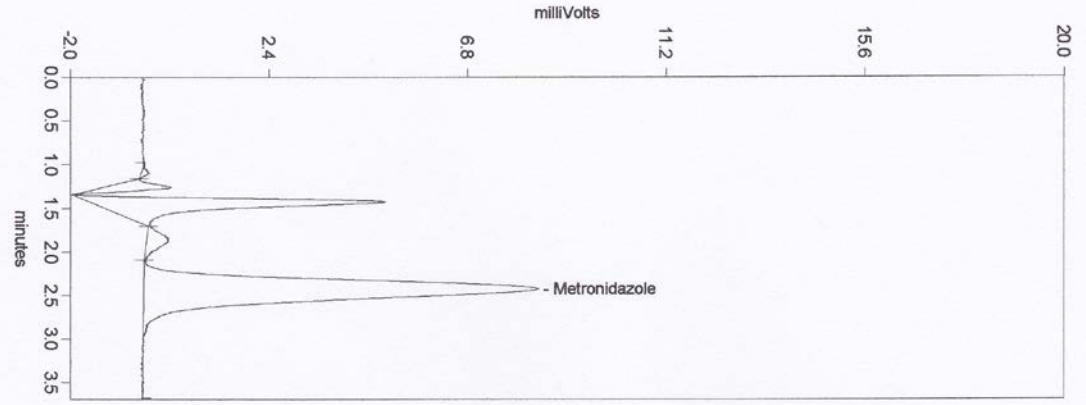
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.42	13.50	224843	0.221
TOTALS :						224843.2	

```
*******************************************************************************
*                                                                             *
*  TIME : 10:03 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   SERUM-1                    CHROMATOGRAM FILE :    SERUD4-1
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE :  FILE
 SAMPLE AMOUNT          1.00                METHOD NAME           : METRONID
 DILUTE       :         1.00                INJ                   :  1 OF  2

 Chromatogram SERUD4-1 recorded at  4:33 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.43	13.80	127416	0.125
TOTALS :						127416.3	

```
*******************************************************************************
*                                                                             *
*  TIME : 10:04 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   SERUM-2                    CHROMATOGRAM FILE :    SERUD4-2
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE :  FILE
 SAMPLE AMOUNT          1.00                METHOD NAME           : METRONID
 DILUTE       :         1.00                INJ                   :  2 OF  2

 Chromatogram SERUD4-2 recorded at  4:39 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



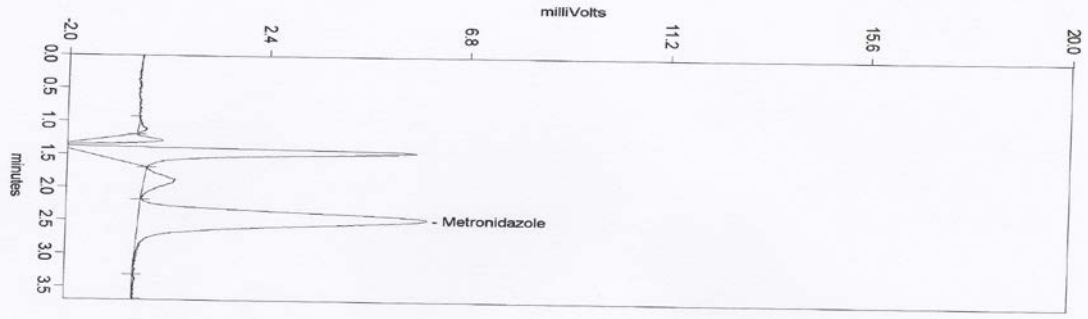
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.42	13.90	132955	0.131
TOTALS :						132955.0	

TIME : 10:03 am DELTA CHROMATOGRAPHY DATA SYSTEM DATE :11/12/2006
EXTERNAL STANDARDS REPORT

SAMPLE NAME : SERUM-1
SAMPLE TYPE : SAMP
SAMPLE AMOUNT 1.00
DILUTE : 1.00

CHROMATOGRAM FILE : SERUD8-1
CHROMATOGRAM SOURCE : FILE
METHOD NAME : METRONID
INJ : 1 OF 2

Chromatogram SERUD8-1 recorded at 4:24 pm on 09/12/2006
Captured by analyst TLN using method : METRONID
Metronidazole content:mg/l



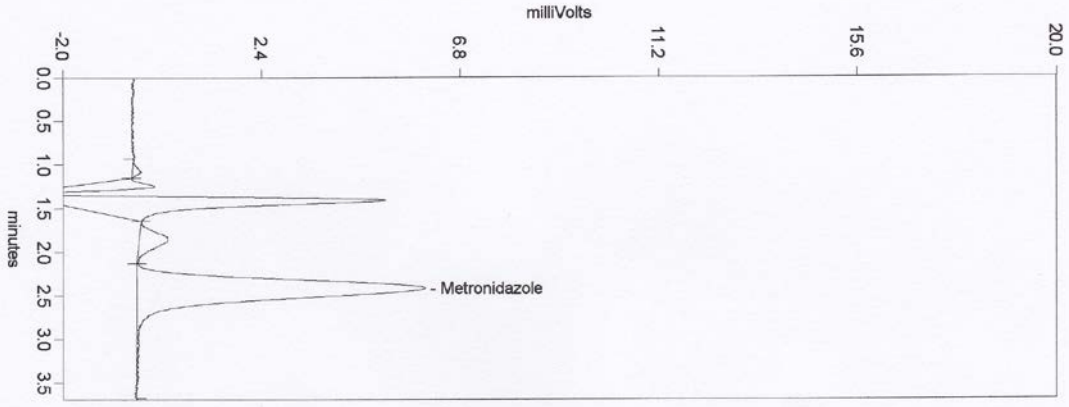
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.46	13.60	94382	0.093
TOTALS :						94381.6	

TIME : 10:03 am DELTA CHROMATOGRAPHY DATA SYSTEM DATE :11/12/2006
EXTERNAL STANDARDS REPORT

SAMPLE NAME : SERUM-2
SAMPLE TYPE : SAMP
SAMPLE AMOUNT 1.00
DILUTE : 1.00

CHROMATOGRAM FILE : SERUD8-2
CHROMATOGRAM SOURCE : FILE
METHOD NAME : METRONID
INJ : 2 OF 2

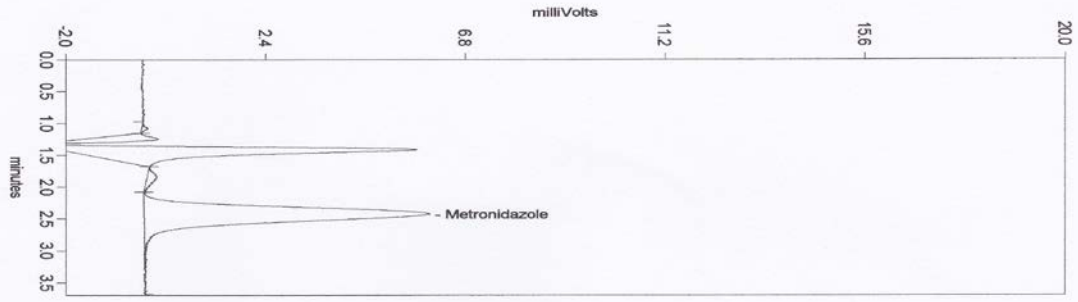
Chromatogram SERUD8-2 recorded at 4:28 pm on 09/12/2006
Captured by analyst TLN using method : METRONID
Metronidazole content:mg/l



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.41	13.70	96265	0.095
TOTALS :						96265.5	

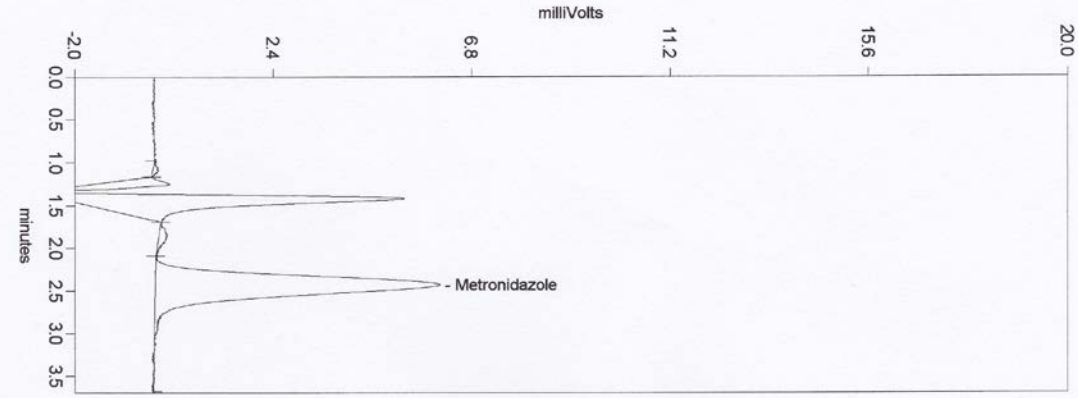
Volunteer E

```
*******************************************************************************
*                                                                             *
*  TIME : 10:19 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                        EXTERNAL STANDARDS REPORT                            *
*******************************************************************************

 SAMPLE NAME :   SERUM-1                    CHROMATOGRAM FILE :    SERUE1-1
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME           : METRONID
 DILUTE      :          1.00                INJ                   :  1 OF  2

 Chromatogram SERUE1-1 recorded at  5:46 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



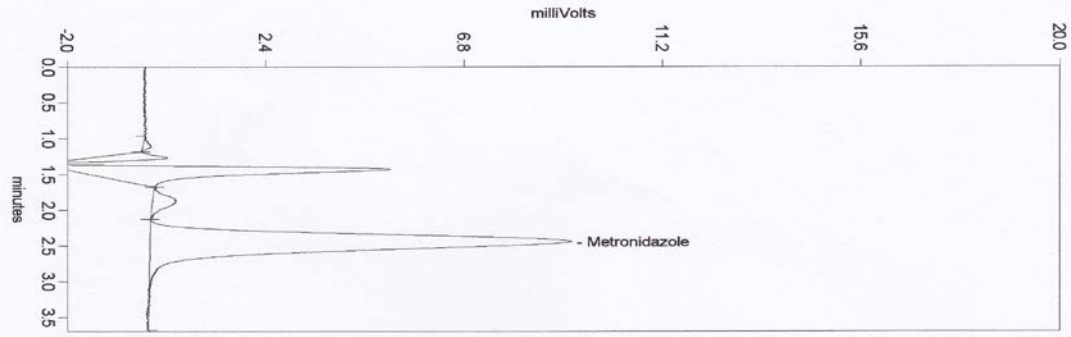
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.43	14.10	98238	0.097
TOTALS :						98237.9	

```
*******************************************************************************
*                                                                             *
*  TIME : 10:19 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                        EXTERNAL STANDARDS REPORT                            *
*******************************************************************************

 SAMPLE NAME :   SERUM-2                    CHROMATOGRAM FILE :    SERUE1-2
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME           : METRONID
 DILUTE      :          1.00                INJ                   :  2 OF  2

 Chromatogram SERUE1-2 recorded at  5:50 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



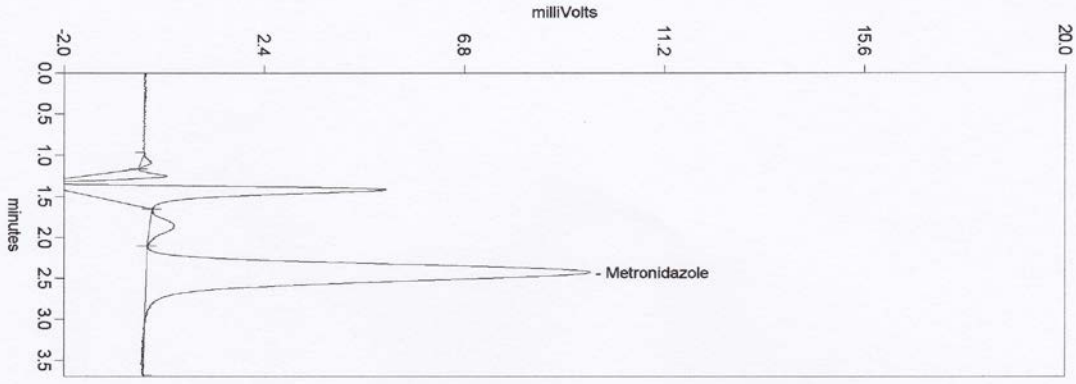
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.43	14.20	97922	0.096
TOTALS :						97922.2	

```
*******************************************************************************
*                                                                             *
*  TIME : 10:19 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   SERUM-1                    CHROMATOGRAM FILE :    SERUE2-1
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME           : METRONID
 DILUTE      :          1.00                INJ                   :  1 OF  2

 Chromatogram SERUE2-1 recorded at  5:37 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



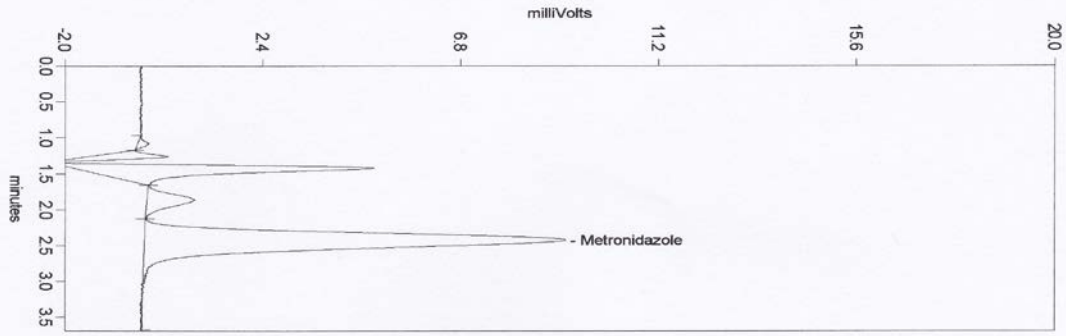
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.44	13.80	141489	0.139
TOTALS :						141489.0	

```
*******************************************************************************
*                                                                             *
*  TIME : 10:19 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   SERUM-2                    CHROMATOGRAM FILE :    SERUE2-2
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME           : METRONID
 DILUTE      :          1.00                INJ                   :  2 OF  2

 Chromatogram SERUE2-2 recorded at  5:41 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



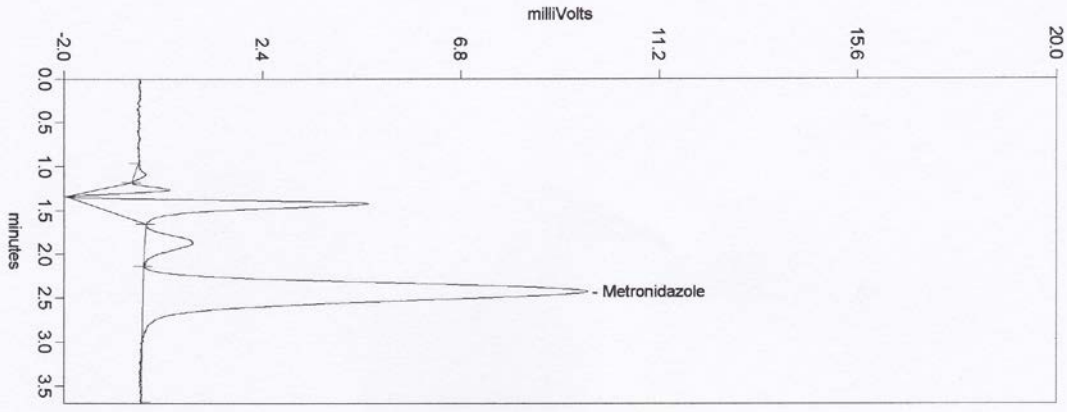
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.43	13.80	146878	0.144
TOTALS :						146877.5	

```
*******************************************************************************
*                                                                             *
*  TIME : 10:19 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   SERUM-1                    CHROMATOGRAM FILE :    SERUE4-1
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME         : METRONID
 DILUTE       :         1.00                INJ                 :  1 OF  2

 Chromatogram SERUE4-1 recorded at  5:29 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



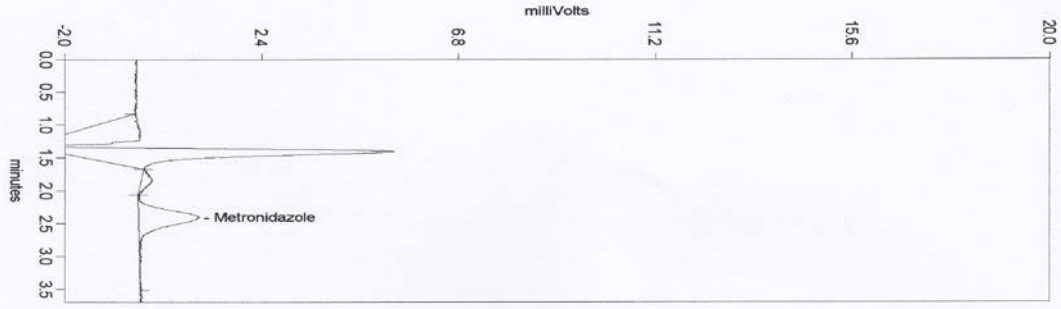
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.42	13.60	138693	0.136
TOTALS :						138692.7	

```
*******************************************************************************
*                                                                             *
*  TIME : 10:19 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   SERUM-2                    CHROMATOGRAM FILE :    SERUE4-2
 SAMPLE TYPE :   SAMP                       CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00                METHOD NAME         : METRONID
 DILUTE       :         1.00                INJ                 :  2 OF  2

 Chromatogram SERUE4-2 recorded at  5:33 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



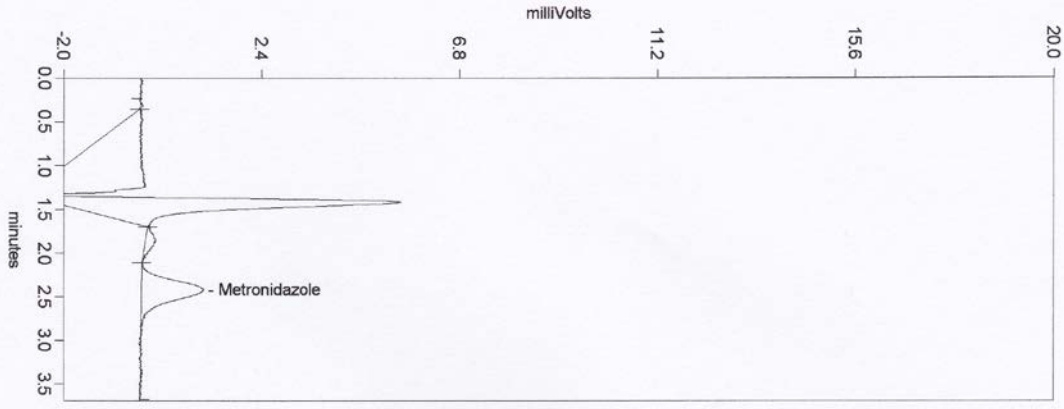
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.42	13.60	145771	0.143
TOTALS :						145771.3	

```
*******************************************************************************
*                                                                             *
*  TIME : 10:19 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   SERUM-1                  CHROMATOGRAM FILE :    SERUE8-1
 SAMPLE TYPE :   SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE      :          1.00              INJ                 :  1 OF  2

 Chromatogram SERUE8-1 recorded at  5:20 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
4	1		Metronidazol	2.40	14.10	21856	0.021
TOTALS :						21855.9	

```
*******************************************************************************
*                                                                             *
*  TIME : 10:19 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :11/12/2006 *
*                            EXTERNAL STANDARDS REPORT                        *
*******************************************************************************

 SAMPLE NAME :   SERUM-2                  CHROMATOGRAM FILE :    SERUE8-2
 SAMPLE TYPE :   SAMP                     CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT          1.00              METHOD NAME         : METRONID
 DILUTE      :          1.00              INJ                 :  2 OF  2

 Chromatogram SERUE8-2 recorded at  5:25 pm on 09/12/2006
 Captured by analyst TLN using method : METRONID
 Metronidazole content:mg/l
```



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
5	1		Metronidazol	2.41	14.30	21987	0.022
TOTALS :						21986.8	

2. Concentration of metronidazole in biological specimens

Table 2: Peak areas and metronidazole concentration (µg/ml) in vaginal specimens

Volunteer/specimen collection time	Area 1	Area 2	Average	Metronidazole concentration (µg/5 ml)*	Metronidazole concentration (µg/ml)
A - 1 h	48756.4	52760.3	50758.35	4.555	0.9110
A - 2 h	75007.6	77857.8	76432.70	5.964	1.1928
A - 4 h	21286.1	25105.2	23195.65	3.043	0.6086
A - 8 h	9046.0	9894.2	9470.10	2.289	0.4578
B - 1 h	35121.4	35197.1	35159.25	3.699	0.7398
B - 2 h	37820.1	34378.3	36099.20	3.750	0.7500
B - 4 h	15471.5	14212.5	14842.00	2.584	0.5168
B - 8 h	24743.3	20541.8	22642.55	3.012	0.6024
C - 1 h	46813.5	49838.2	48325.85	4.421	0.8842
C - 2 h	19611.4	18711.8	19161.60	2.821	0.5642
C - 4 h	8025.8	12926.7	10476.25	2.345	0.4690
C - 8 h	8812.4	11465	10138.70	2.326	0.4652
D - 1 h	13531.1	18425.6	15978.35	2.6470	0.5294
D - 2 h	55858.2	53739.5	54798.85	4.776	0.9552
D - 4 h	61624.1	76742.6	69183.35	5.566	1.1132
D - 8 h	46527.3	45530.2	46028.75	4.295	0.8590
E - 1 h	30341.1	26602.8	28471.95	3.332	0.6664
E - 2 h	25604.4	22580.9	24092.65	3.092	0.6184
E - 4 h	64668.1	61986	63327.05	5.244	1.0488
E - 8 h	17810.9	19082.7	18446.80	2.7820	0.5564

*freeze-dried vaginal secretion was dissolved in 5 ml mobile phase solvent

Table 3: Peak areas and metronidazole concentration (µg/ml) in serum specimens

Volunteer/specimen collection time	Area 1	Area 2	Average	Metronidazole concentration (µg/ml)
A - 1 h	40954.0	42216.7	41585.35	6.025
A - 2 h	93013.6	97299.8	95156.70	6.991
A - 4 h	145222.1	146396.3	145809.20	9.770
A - 8 h	111208.2	110965.7	111086.95	7.865
B - 1 h	181434.2	184426.9	182930.55	11.807
B - 2 h	165070.9	166829.0	165949.95	10.827
B - 4 h	178835.6	188978.8	183907.20	11.582
B - 8 h	106762.9	107613.7	107188.30	7.628
C - 1 h	117463.0	118520.5	117991.75	8.244
C - 2 h	215559.8	224803.4	220181.60	13.851
C - 4 h	132740.7	134930.0	133835.35	9.113
C - 8 h	41602.0	44193.0	42897.5	4.124
D - 1 h	139138.0	131621.7	135379.85	9.198
D - 2 h	233206.4	224843.2	229024.80	14.336
D - 4 h	127416.3	132955.0	130185.65	8.913
D - 8 h	94381.6	96265.5	95323.55	7.000
E - 1 h	98237.9	97922.2	98080.05	7.151
E - 2 h	141489.0	146877.5	144183.25	9.681
E - 4 h	138692.7	145771.5	142232.10	9.5740
E - 8 h	21855.9	21986.8	21921.35	2.9730

APPENDIX G

DNA isolation and gel electrophoresis solutions

1. **Phosphate-Buffered Saline (PBS), pH 7.4**

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Distilled water	800 ml

The solution was adjusted to a pH of 7.4 using I M HCl, made up to a volume of 1 L and autoclaved.

2. **TSE, pH 8**

Tris-HCl (20 mM)	1.58 g
NaCl (100 mM)	2.92 g
EDTA (50 mM)	9.36 g

The solution was adjusted to a pH of 8 with 1 M NaOH and autoclaved.

3. **Proteinase K (20 mg/ml)**

20 mg proteinase K (Boehringer Mannheim, Germany) was dissolved in 1 ml sterile distilled water, aliquoted in 50 µl volumes and stored at –20^{o}C.

4. Rnase A (10 mg/ml)

Sodium acetate	0.01 M
Distilled water	15 ml

0.027 g of sodium acetate was dissolved in 15 ml distilled water, the pH was adjusted to 5.2, and the volume was made up to 20 ml with distilled water.

10 mg Rnase A (Boehringer Mannheim, Germany) was dissolved in 1 ml sodium acetate. The enzyme preparation was heated to 100^{o}C for 15 minutes and allowed to cool slowly to room temperature. 50 µl volumes were aliquoted into Eppendorf tubes and stored at –20^{o}C.

5. 1 M Sodium citrate

Sodium citrate	147.05 g
Distilled water	500 ml

Dissolve and autoclave.

6. 1M Tris-HCl, pH 7.6

Tris base	24.22 g
Distilled water	160 ml

pH was adjusted to 7.6 with approximately 12 ml of concentrated hydrochloric acid. The volume was adjusted to 200 ml, dispensed and autoclaved.

7. 10 mM TE Buffer, pH 7.6

Tris-HCl, pH 7.6	5 ml
EDTA	0.1861 g
Distilled water	300 ml

The EDTA was dissolved and the pH was adjusted to 7.6, using 1 M HCL. The volume was adjusted to 500 ml and autoclaved for 20 minutes.

8. 5x TBE, pH 8.5

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA, pH 8	20 ml

The solution was adjusted to a pH of 8.5, made up to a volume of 1 L and autoclaved.

9. Ethidium bromide (10 mg/ml)

Add 1 g of ethidium bromide (Sigma-Aldrich, USA) to 100 ml of sterile distilled water. Stir on a magnetic stirrer for several hours until the dye has dissolved. The container containing the ethidium bromide was wrapped in aluminium foil and stored at room temperature.

10. Gel loading buffer

Glycerol	50g
Tris-HCL ph7.5	5 ml of 1M Tris HCL, ph7.5
EDTA	1ml of 500 mM EDTA
Bromophenol blue	0.05 g

RNase	300 µl of 10 mg/ml
Distilled water	to 100 ml

Boil for 15 minutes at 100^{o}C. Cool overnight at room temperature. Dispense into Eppendorf tubes and store at -20^{o}C.

11. Agarose gel

Agarose was added to 0.5x TBE buffer and dissolved by heating in a microwave. Ethidium bromide (0.5 µg/ml) (Sigma-Aldrich, USA) was added to the solution after cooling to 50^{o}C. This agarose solution was poured into a gel mould and, a gel comb was placed at one end of the gel. The agarose was allowed to set for 30-45 minutes at room temperature before removing the comb. The gel was then transferred to an electrophoresis tank.

APPENDIX H

Simpson's Index of Diversity (Hunter and Gaston, 1988)

Simpson's Index of Diversity, D, was calculated as follows:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1)$$

Where s is the number of types; nj is the number of strains falling into the j type and N is the size of the sample population. This statistic gives the probability that two randomly selected strains will belong to different types. A value of D = 0 would indicate that all the population members are identical and D = 1 would indicate that all the population members differ from one another.

Calculation: Sample size: 100
No of types: 4

Strain distribution: Type A = 7
Type B = 28
Type C = 47
Type D = 18

Index of diversity
N = 100
S = 4

$$D = 1 - \frac{1\,(7X6+28X27+47X46+18X17)}{100 \times 99}$$

$$1 - \frac{1\,(42+756+2162+306)}{9900}$$

$$1 - \frac{3266}{9900}$$

$$1 - 0.330$$

D = 0.67

APPENDIX I

PCR-RFLP Gels

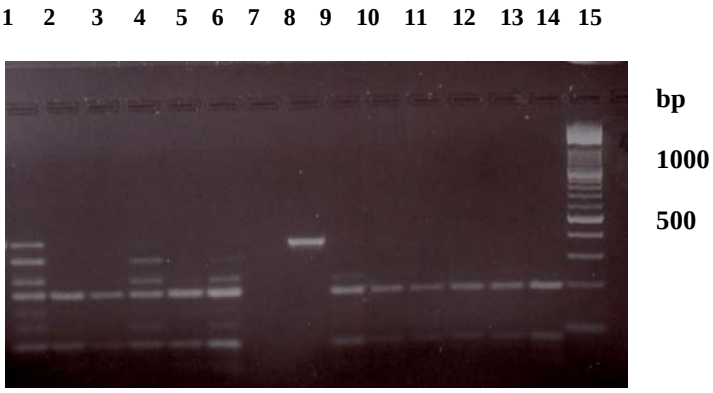


Figure 2: *Acs1* PCR-RFLP patterns of the PCR amplicon. Lanes 1-15: ATCC 50138, KW12, STD2, KW13, STD3, CSW3, blank, uncut PCR amplicon, STD4, KW14, CSW4, KW15, KW16, CSW5, DNA molecular weight marker XIV

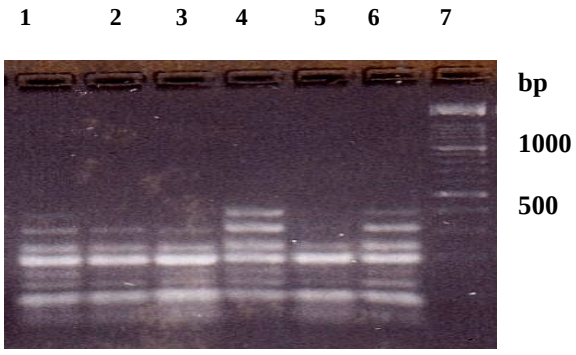


Figure 3: *Acs1* PCR-RFLP patterns of the PCR amplicon. Lanes 1-7: KW17, CSW6, STD5, STD6, KW18, STD7, DNA molecular weight marker XIV

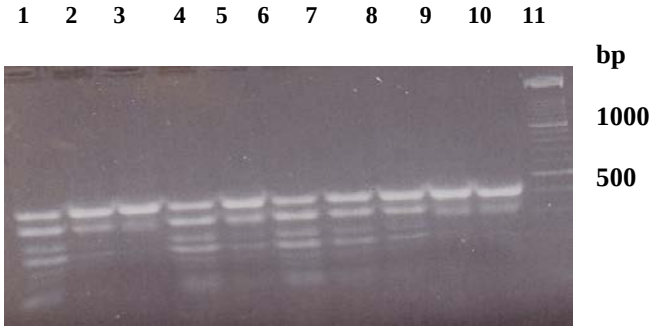


Figure 4: *Acs1* PCR-RFLP patterns of the PCR amplicon. Lanes 1-11: ATCC 50138, KW19, KW20, CSW7, CSW8, STD8, STD9, KW21, KW22, KW23, DNA molecular weight marker XIV

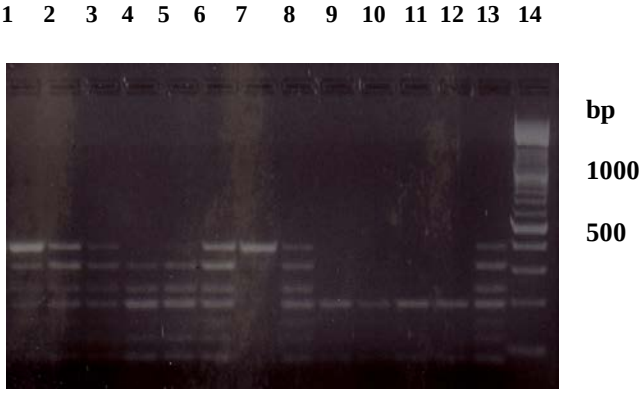


Figure 5: *Acs1* PCR-RFLP patterns of the PCR amplicon. Lanes 1-14: KW24, KW25, CSW9, KW26, KW27, KW28, uncut PCR amplicon, CSW10, KW29, CSW11, CSW12, CSW13, ATCC 50138, DNA molecular weight marker XIV

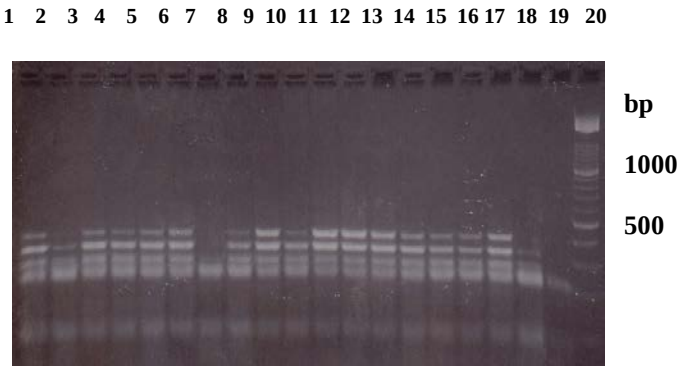


Figure 6: *Acs1* PCR-RFLP patterns of the PCR amplicon. Lanes 1-20: ATCC 50138, KW30, KW31, KW32, CSW14, KW33, CSW15, KW34, KW35, KW36, CSW16, CSW17, KW37, KW38, CSW18, CSW19, CSW20, KW39, KW40, DNA molecular weight marker XIV

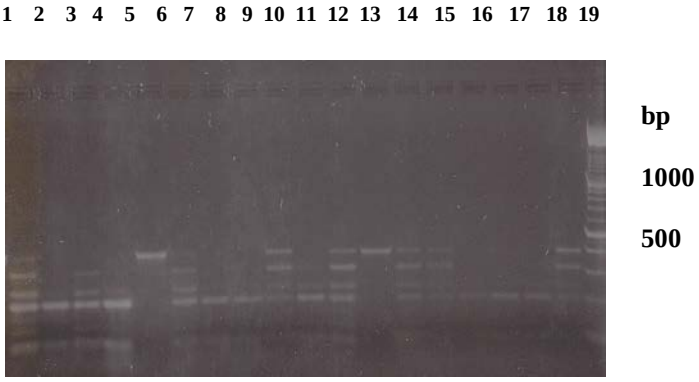


Figure 7: *Acs1* PCR-RFLP patterns of the PCR amplicon. Lanes 1-19:, ATCC 50138, KW41, KW42, KW43, uncut PCR amplicon, KW44, CWS21, CWS22, KW45, KW46, KW47, uncut PCR amplicon, CSW23, CSW24, CSW25, CSW26, KW48, KW49, DNA molecular weight marker XIV

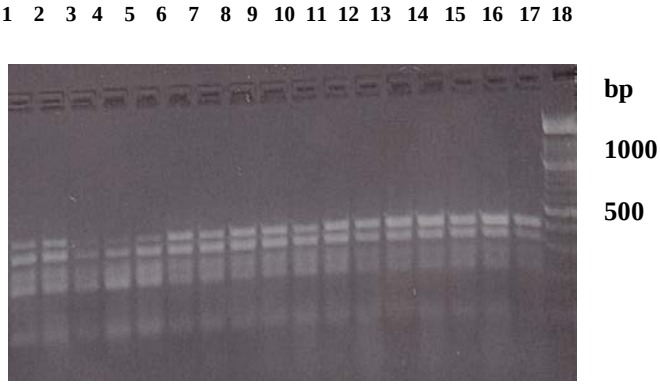


Figure 8: *Acs1* PCR-RFLP patterns of the PCR amplicon. Lanes 1-18:, KW50, CSW27, KW51, KW52, KW53, CSW28, KW54, KW55, KW56, CSW29, CSW30, KW57, KW58, CSW31, KW59, KW60, ATCC 50138, DNA molecular weight marker XIV