The Preparation and use of Tc-99m Metronidazole for Cervical Cancer Imaging

A dissertation submitted by

Amanda Henrietta Mdlophane

in partial fulfilment of the requirements for the degree

Master of Science in Medicine (Pharmacy)

of the

School of Health Care Sciences

Faculty of Health Science

Supervisor: Dr. B Summers

Co-supervisor: Prof. M Sathekge

Department of Pharmacy

University of Limpopo (Medunsa Campus)

2011

DECLARATION

I, Amanda Henrietta Mdlophane, hereby declare that the work on which this study is based is original, except where acknowledgements indicate otherwise.

This dissertation is submitted for the degree Master of Science in Medicine (Pharmacy) at the University of Limpopo, Medunsa Campus. Neither the whole work nor any part of it has been submitted before for any degree or examination at this or any other university.

Signed……..…on the……day of……..

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

This has been a very long road with multiple challenges which ultimately changed the course of this study, a character building experience. My endless gratitude goes first to YAWEH, God Almighty who has given me strength from day to day to complete successfully what I had embarked on. The following people have given me support, their time and expertise to create this precious document...

1. To my family. You are everything to me, you anchored me and encouraged me to keep on going on. Your endless support is forever appreciated. My friends, you were like the extension of my family. You reminded me that I'll reap great rewards from all the challenges I faced. Thank you, for being present and listening.

2. Dr B. Summers. My supervisor and mentor, thank you so much for the time you put into this project even though you have so many other things to do. Your encouragement never to give-up kept me going.

3. Prof M. Sathekge. In your very busy schedule you managed to squeeze me in, even without an appointment. Thank you for imparting your expertise.

4. Dr O. Knoesen. There are no words to describe how much I appreciate the time and effort you put into making this project happen. You sacrificed endless working hours driving back and forth without complaint. I'm forever indebted to you.

5. Dr A. Gutta. Thank you also for showing interest in this project, for clarifying the complex issues and also for opening my eyes to the realities of research.

6. Participants. Thank you for allowing us to use your condition as a learning curve and for allowing us to add to the knowledge database of the complexities of treating cervical cancer.

7. Department of Nuclear Medicine, UP/SBAH. Thank you for your time and for training me at the hot laboratory. For answering my endless questions about each and everything and for welcoming me as one of your own. Thank you also for providing the facility to conduct the research.

8. Dr M. Soni. Thank you for running around helping me to locate the patient files and the different departments of SBAH, Tshwane District Hospital and the Medical Campus of the University of Pretoria.

9. Prof M. Bida. Thank you for your input and expertise in the analysis of the histology report. Now I know what's positive and what's negative.

10. The Medical Research Council. Thank you for financially supporting this study.

11. Department of Pharmacy, Medunsa Campus. Thank you for supporting me financially, providing facilities for me to work and for the endless advice. Most importantly thank you for training me to be a problem solver.

12. NTP Radioisotopes (Pty) Ltd. Thank you for providing the facilities and equipment to do further analyses and for allowing Dr Knoesen to lend a hand.

13. To all who donated apparatus, who gave advice, your input made a significant difference.

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

AHFS	American Hospital Formulary Service
BP	British Pharmacopoeia
cGRPP	current Good Radiopharmacy Practice
CIN	Cervical Intraepithelial Neoplasia
CT	Computed Tomography
DAD	Diode Array Detector
DoH	Department of Health
EC-MN	Ethylene-dicysteine Metronidazole
EPO	Erythropoietin
FDA	Food and Drug Administration
FDG	Fluoro-deoxyglucose
FIGO	International Federation of Gynaecology and Obstetrics
^{18}FMISO	^{18}Fluoro-misonidazole
GIT	Gastro-intestinal Tract
HIF-1	Hypoxia Inducible Factor -1
HIV/AIDS	Human Immunodeficiency Virus/ Acquired Immune Deficiency Syndrome
HPLC	High Performance Liquid Chromatography
HPV	Human Papilloma Virus
HSIL	High Grade Squamous Intra-epithelial Lesion
IP	International Pharmacopoeia
ITLC-SG	Instant Thin Layer Chromatography with silica gel
LLETZ	Loop Excision of the Transformation Zone
MISO	Misonidazole

MREC	Medunsa Research and Ethics Committee
MRI	Magnetic Resonance Imaging
NTP	Nuclear Technology Products (Pty) Ltd.
PET	Positron Emission Tomography
QA	Quality Assurance
QC	Quality Control
Rf	Retardation factor
SBAH	Steve Biko Academic Hospital
TLC	Thin Layer Chromatography
UP	University of Pretoria
UPREC	Research and Ethics Committee of the University of Pretoria

ABSTRACT

Introduction: Non-invasive detection of tumour hypoxia theoretically adds value to the outcome of treatment; however the practical aspect of using ^{99m}Tc-EC-MN in cervical cancer remains un-attempted. ^{99m}Tc-EC-MN has been used to indirectly detect hypoxia in many tumours (head and neck) and other hypoxic states such as strokes and MI. This study aims to determine the value of using this tracer in early stage cervical cancer.

Objectives: This study aimed to investigate the use of ^{99m}Tc-EC-MN to determine the degree of hypoxia in cervical cancer. The original study design was to determine whether SPECT with ^{99m}Tc-EC-MN would detect hypoxic cervical cancer lesions and compare the results with the histological report. The practice of safe handling of radiopharmaceuticals and gaining knowledge in conducting research formed part of the secondary objective of the study. Due to circumstances beyond the control of the researcher, the focus of the study changed from a clinical to a chemistry-based project.

Method development: Safety of EC-MN was tested through determination of the labelling efficiency with pertechnetate initially by ITLC-SG. Ethyl acetate, ethanol, saline and acetone were selected to develop ^{99m}Tc-EC-MN chromatograms to identify the system which best displays separation. Radio-ITLC displayed multiple peaks due to high residual activity in ethyl acetate- and acetone-developed scans. Saline- and ethanol-developed scans showed better separation of ^{99m}Tc-EC-MN but separation from free pertechnetate was difficult. Radio HPLC coupled with a diode array detector was used to successfully separate the labelled product, ^{99m}Tc-EC-MN from free pertechnetate, thereby achieving good radiolabelling.

Clinical application: After the relative safety of the product was established, it was injected IV in the selected patient who had early stage cervical carcinoma. Clinical examinations which included pre-operative WBC, ultrasonography of the kidneys and bladder, and chest x-rays were performed. Histological analysis was performed after surgery and gave results that were insufficient to conclude the absence or the presence of tumour hypoxia. Detection of ^{99m}Tc-EC-MN was analysed from blood-flow and -pool images, thyroid and pelvic static, SPECT, and WBS images obtained from a gamma scintillation camera. Faint hot spots consistent with low levels of free pertechnetate were detected in the salivary glands. Hot areas which paralleled the bio-distribution of the ^{99m}Tc-EC-MN were also detected in the thyroid, liver, intestines, kidneys, and bladder. There was no tracer detection in the pelvic area.

Conclusions: Experience was gained in QC procedures and aseptic preparation of radiopharmaceuticals, and in conducting and co-managing a chemical and clinical based research. Radiochemically related findings demonstrated that tin (II) chloride can be solubilised in water; ^{99m}Tc-EC-MN migrates with the solvent front in saline and ethanol developed ITLSG scans; and ITLC cannot sufficiently separate ^{99m}Tc-EC-MN from free pertechnetate. Successful labelling of EC-MN was confirmed by scintigraphy and showed tracer distribution that parallels those previously described. Successful labelling of EC-MN with ^{99m}Tc can be achieved up to two years after kit manufacture given appropriate storage conditions for the EC-MN. The hypoxic status of the tumour remained inconclusive; therefore the prognostic impact of ^{99m}Tc-EC-MN in cervical cancer remains unknown.

Recommendations: Product stability and potential expiry should be available for all products, even in the developmental stages and particularly for clinical trials. A simple QC method to separate ^{99m}Tc-EC-MN from free pertechnetate should be developed. Further studies are required in order to confirm the efficacy of ^{99m}Tc-EC-NM in determining tumour hypoxia in cervical cancer. If a suitable animal model is not available, patients with known cancer tissue hypoxia should be evaluated and compared with those who are non-hypoxic.

CHAPTER 1
INTRODUCTION

1.1 INTRODUCTION

The chapter gives the overview of the research project. The chapter includes the background of the study, the problem statement and the importance of conducting a study of this kind. The aim and objectives are defined.

1.2 BACKGROUND AND RATIONALE FOR THE STUDY

Cervical cancer is the second most common malignancy in women worldwide, and it remains a leading cause of cancer-related death for women in developing countries. In 2002, it was estimated that 493 000 new cases of cervical cancer were diagnosed globally and 247 000 women died in that year (Parkin *et al*., 2005). Approximately 85 to 90% of cervical cancers are squamous cell carcinomas, and most of the remaining 10 to 15% are adenocarcinomas (Landoni *et al*., 1997; Waggoner, 2003).

The definitive treatment and prognosis for patients with cervical cancer is markedly affected by the extent of disease at the time of diagnosis. When cancer is confined to the cervix or adjacent vagina, radical surgery or radiotherapy alone could obtain similar survival rates (Landoni *et al*., 1997; Waggoner, 2003). When there is regional spread or bulky (≥4 cm) tumour, multimodality therapy will be necessary (Rose *et al*., 1999a). As a solid tumour like cervical cancer grows, some cells inevitably become physically separated from the native tissue's nutrient vascular stroma, and delivery of nutrients and oxygen to those cells becomes limited to diffusion (Kerbel, 2000; Jemal *et al*., 2002). The end result is metabolic stress and cellular hypoxia – conditions which are thought to be major stimuli underlying the recruitment of additional blood vessels (Richard, Berra & Pouysségur, 1999). Hypoxia induces changes in transcriptional regulation that serve to alter cellular metabolism (i.e. there is a, shift to glycolytic pathways). These changes promote the ingrowth of immature, architecturally deranged and highly permeable blood vessels that facilitate the passage of tumour cells into the circulation (Hanahan & Folkman, 1996). Thus a cancer's response to hypoxia not only sustains tumour growth and survival, but through angiogenesis fosters invasion and metastasis. Indeed, hypoxic tumours have been reported to have a predilection for tissue invasion and metastasis (Brizel *et al*., 1996; Höckel *et al*., 1996).

A role for a hypoxic microenvironment in the pathogenesis and progression of human cancer was first proposed in 1953, when well-oxygenated tumour cells were found to exhibit 3-fold greater response to radiotherapy when compared to anoxic cells (Gray, Conger & Ebert, 1953). The reasons why tumours become hypoxic remain unclear, but multiple mechanisms are likely to contribute to their development. These mechanisms include unrestrained growth and accelerated oxygen consumption by tumour cells; poor lymphatic drainage of tumours resulting in high interstitial pressures, vascular collapse, and low pH; and intratumoural shunting of oxygen-rich blood through immature vasculature (Giatromanolaki & Harris, 2001).

Hypoxia is thus an important factor in many pathological processes, including tumour formation, where it has been associated with resistance to radiotherapy, malignant progression, and metastasis. Although hypoxia is toxic to both cancer and normal cells, cancer cells undergo genetic and adaptive changes that allow them to survive and even proliferate within a hypoxic environment. These processes contribute to the malignant phenotype and to aggressive tumour behaviour. Other than the direct measurement of oxygen tension in human tumours, which can be performed with polarographic oxygen electrodes, which is invasive and not routinely used, there is no validated method which is currently used to measure hypoxia and correlate outcome (Höckel *et al*., 1993; Höckel *et al*., 1996).

Positron emission tomography (PET) is an interesting and promising diagnostic modality for hypoxia imaging of tumour tissue. Several tracers have been developed for this purpose. Radiolabelled Fluoromisonidazole (^{18}FMISO) remains the most extensively studied hypoxia tracer, both in humans and animals (Koh, Rasey & Evans, 1992; Nunn, Linder & Strauss, 1995; Rasey *et al*., 1996). Previous results for imaging hypoxia using ^{18}FMISO showed the regional distribution of tumour hypoxia, expressed as fractional hypoxic volume. However, ^{18}FMISO has major disadvantages – i.e. slow clearance kinetics and high lipophilicity (Rasey *et al*., 1990; Nunn, Linder & Strauss, 1995). Moreover, PET imaging hardware is expensive with limited availability in the developing world, whereas conventional Gamma camera/SPECT imaging is more accessible. Hence a SPECT (single photon emission tomography) tracer for imaging hypoxia is more practical and desirable in the developing world (Gutta, 2011).

Nitroimidazole compounds are enzymatically reduced within viable hypoxic cells. This mechanism is well understood through numerous *in vitro* and *in vivo* studies from the past three decades (Chu *et al*., 2004).

Technetium-99m (^{99m}Tc) has favourable physical characteristics, a low price, and is readily available, unlike ^{18}F. Other attractive properties of ^{99m}Tc include gamma ray emission between 100-250KeV, a short half-life that allows for practicality, offers a high target /background ratio for good image contrast and it has the ability to bind with a variety of ligands and remain stable *in vivo* (University of Limpopo (Medunsa Campus), 2009).

Recently, ^{99m}Tc-ethylene dicysteine-metronidazole (EC-MN) has been developed to assess the hypoxic components of cerebrovascular accident, myocardial infarction, and various tumours (Yang *et al*., 1999). Similar to other nitroimidazole derivatives such as ^{18}FMISO, metronidazole is trapped within hypoxic cells. It has also been successfully used in humans to localise in brain tissue that is hypoxic but viable after ischaemic stroke and the higher the uptake of ^{99m}Tc-EC-MN, the better the neurological outcome (Song *et al*., 2003).

1.3 STUDY PROBLEM

Hypoxia has a negative effect on anti-cancer treatment (Brown, 1999). Radiotherapy is a major treatment modality for advanced cervical carcinomas and requires free radicals from oxygen to destroy target cells. Cells in hypoxic areas have been found to be resistant to radiation-induced cell death (Bachtiary, *et al*., 2003). Even-though radiotherapy is the major treatment modality, little is known about the prognostic effects of hypoxia on operable patients. The gold standard for measuring hypoxia in tumours is the invasive technique of using computerized oxygen-sensitive electrodes. This method is cumbersome and expensive and has not become a general clinical tool for hypoxia measurement (Höckel *et al*., 1993; Höckel *et al*., 1996). Non-invasive functional imaging with ^{99m}Tc-ethylene dicysteine-metronidazole (EC-MN) may help to address this poor record.

1.4 AIM OF THE STUDY (AS STATED IN THE PROPOSAL)

To determine the prognostic impact of ^{99m}Tc labelled metronidazole in cervical cancer.

1.5 OBJECTIVES OF THE STUDY (AS STATED IN THE PROPOSAL)

The objectives of the study were as follows:

- To determine whether SPECT with ^{99m}Tc-EC-MN can detect hypoxic tissues in cervical cancer;
- To determine the diagnostic value of SPECT with ^{99m}Tc-EC-MN in characterizing cervical cancer lesions and compare results of SPECT with ^{99m}Tc-EC-MN with

existing markers of hypoxia and poor prognosis on immunohistochemistry, clinical staging and MRI; and

- To practice safe handling of radiopharmaceuticals and to gain experience in the co-management of a clinical study as well as a working knowledge of the research process.

1.6 IMPORTANCE OF THE STUDY

Tumour hypoxia in several solid cancers, notably those of uterine cervix, head and neck, and lung, has been recognized for many years to be important in determining response to radiotherapy, because tumour cells become radiotherapy-resistant at low oxygen tensions (Brown, 1999). Direct measurement of oxygen tension in human tumours can be performed with polarographic oxygen electrodes (Eppendorf GmbH, Hamburg, Germany). The pre-treatment oxygen tension assessed with this method has been shown to predict response to radiation therapy and survival in patients with cervical cancer (Höckel *et al*., 1993; Höckel *et al*., 1996). However, this method is invasive and technically demanding and thus has not been widely used in clinical practice. Accordingly, there has been substantial interest in the development of noninvasive imaging methods for assessing tumour hypoxia.

The most widely studied method involves the use of positron emission tomography (PET) with the radiolabeled nitroimidazole derivative, ^{18}FMISO (Koh, Rasey & Evans, 1992). This compound is reduced enzymatically and trapped within hypoxic cells. However, because of the relatively low uptake of ^{18}FMISO in hypoxic tissues, its clinical application has had limited success, furthermore, PET hardware is costly and therefore limits its availability in developing countries. Hence ^{99m}Tc-EC-MN which has favourable physical characteristics, a low price, and is readily available, unlike ^{18}F, could offer a solution. Several authors concluded that SPECT imaging with ^{99m}Tc-EC-MN appears to be safe and feasible in patients with cerebrovascular accident, myocardial infarction, and various tumours (Yang *et al.,* 1999). This procedure can be compared with other methods which include immunohistochemistry for hypoxic markers, histological assessment for vascularity and MRI vascular measures.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

In this chapter, the literature is reviewed and the collected knowledge divided into manageable, sequential sections. Cervical cancer has been extensively studied; aetiology, symptoms and signs, diagnosis and the available treatment options and preventative measures have been described. The complications, which are the focus of this study, have also been well described, with many attempts to bypass them. Radiopharmaceuticals show great promise in resolving this problem as explained in section 2.3.4.

2.2 CERVICAL CANCER

2.2.1 Aetiology

In underdeveloped countries, cervical cancer is caused primarily by the human papillomavirus (HPV), a sexually transmitted infection (Bosch *et al*., 1995; Chichoreon *et al*., 1998; Ngelangel *et al*., 1998). The main HPV gene subtypes that have been implicated are E6 and E7. Denny (2010) argues that Type 16 and 18 are the “high-risk viruses” associated with carcinoma of the cervix. Waggoner (2003) reports that other risk factors include sexual activity at <16 years of age, multiple sexual partners within the same period, genital warts, HIV positive status and immunosuppression due to other causes, such as drugs.

The morbidity and mortality due to HIV/AIDS in South Africa immediately demands attention and awareness of the importance of early diagnosis of cervical cancer. Smoking has also been implicated as a dose-related risk factor, with particular significance for invasive cervical cancer and cervical dysplasia (Slattery *et al*., 1989; Daly *et al*., 1998; Kjellberg *et al*., 2000). Research has shown that when a person has more than one risk factor, it increases their chance of developing cervical cancer (Denny, 2010).

2.2.2 Pathophysiology

Cervical carcinoma tumour cells have been found to occur as a result of gene expression of E6 and E7 subtypes of HPV (Grigsby *et al*., 2006). Through gene expression, there is a histological change from normal cells to invasive cancer. There is initially minimal cervical

dysplasia in the lower third of the epithelium which can progress to a stage in which the full thickness of epithelium contains these abnormal cells.

The cancer is termed 'invasive' when the basement membrane has been penetrated and the stroma invaded. When these genes are not suppressed, the tumour continues to grow and metastasises firstly to the local region (pelvic lymph nodes) then to the para-aortic region. At this stage prognosis may still be favourable but further involvement of the supraclavicular lymph nodes and other distant sites decreases survival rates (Grigsby *et al*., 2006).

In any form of cancer, the initial step involves abnormal cellular changes, usually over many years. These abnormal changes are not cancerous but are pre-cancerous. In cervical cancer the pre-cancerous change begins in epithelial cells and has recently been termed a squamous intraepithelial lesion (SIL), describing the area of abnormal cells, its location and extent of invasion (Denny, 2010).

SIL is categorised into two groups: lower grade SIL and higher grade SIL. In a lower grade lesion there are initial changes in cell shape, size and number. The tumour may regress or develop into a higher grade lesion, which refers to an increased number of the pre-cancerous cells. Lower grade SIL was previously known as mild dysplasia or cervical intraepithelial neoplasia grade 1 (CIN I) (Denny, 2010).

2.2.3 Epidemiology / Prevalence

Cancer of the uterine cervix is reported to have the highest mortality amongst cancer patients in developing countries as opposed to developed countries (Waggoner, 2003). Parkin *et al.* (2002) observed that approximately 80% of all cases emerge in underdeveloped countries.

In South Africa alone, statistics estimate that one in every 41 women will develop cervical cancer in their lifetime (DoH, n.d.), while some statistical data report that one in 34 women is affected (Denny, 2010). Cervical cancer is the second most common cancer affecting all South African women after breast cancer. In the Negroid population, it is the leading form of cancer, often diagnosed at a very advanced stage (DoH, n.d.).

It takes approximately 10-20 years for precursors to become invasive. However, younger women, below 50, who present with low grade SIL, usually regress to normal whereas in older women the opposite is true (DoH, n.d). It is most unfortunate that with the ever escalating growth of HIV infections being reported, younger women are at high risk.

2.2.4 Signs and symptoms

Carcinoma of the cervix, at the early stage, is asymptomatic. As it spreads beyond the epithelium the symptoms begin to appear, mainly vaginal bleeding. Bleeding may occur between menstrual periods, after sexual intercourse or douching. It is often distinct in women who have reached menopause. Another common symptom often mentioned is increased vaginal discharge that has a strong odour (Denny, 2010).

2.2.5 Diagnosis

The implemented screening programme in South Africa aims to diagnose at least 70% of women, nationally, within the target age group (30 years and above). The three main objectives of the programme are: to reduce the incidence of cervical cancer by targeting the disease at its pre-invasive stage, to reduce “the morbidity and mortality associated with” carcinoma of the cervix and ultimately to reduce “the excessive expenditure of scarce health funds currently spent on the treatment of invasive cancer of the cervix” (DoH, n.d.).

Analysis of a Papanicolaou (Pap) smear has been the standard diagnostic method applied in the health institutions of the public sector in South Africa. Pap smears are analysed for cervical abnormalities preceding invasive cancer to identify a squamous intraepithelial lesion (SIL) or formerly, a cervical intraepithelial neoplasia (CIN). These precursors are grouped into mild CIN I (low grade SIL), moderate CIN II and severe forms CIN III (high grade SIL) (DoH, n.d.).

A lower-grade lesion is one involving dysplasia in the lower third of the epithelium (depth) of the cervix. At this stage the patient is only required to return for repeat biopsies or smears. If, however, there are lesions on her return, more tests are performed (Denny, 2010).

A higher grade lesion includes the middle third of the epithelial thickness (CIN II) and the upper third (CIN III). A lesion that has been graded as HSIL on a Pap smear requires further investigation. When the lesion has invaded the tissue beneath the epithelial lining, the lesion is termed invasive cancer (Denny, 2010).

2.2.5.1 Tumour Staging

Staging determines the extent of invasion of the cancer and the presence of metastases. The staging of the lesion by International Federation of Gynaecology and Obstetrics (FIGO) staging criteria uses a clinical basis (more detail in Table 2.1 below). Clinical examinations include blood and urine tests, cytoscopy and proctosigmoidoscopy in addition to histological assessment of the biopsy specimen taken from the smear (Makwela, 2010).

Table 2.1: FIGO staging for cervical cancers

STAGE	DESCRIPTION
Stage 0	Carcinoma-in-situ, intraepithelial carcinoma
Stage I	Invasive carcinoma strictly confined to cervix
Stage IA	Invasive carcinoma identified microscopically (all gross lesions, even with superficial invasion, should be assigned to stage IB)
Stage IA1	Measured invasion of stroma 3.0 mm or less in depth and no wider than 7.0 mm
Stage IA2	Measured invasion of stroma more than 3.0 mm but no greater than 5.0 mm in depth and no wider than 7.0 mm
Stage IB	Preclinical lesions greater than stage IA or clinical lesions confined to cervix
Stage IB1	Clinical lesions of 4.0 cm or less in size
Stage IB2	Clinical lesions more than 4.0 cm in size
Stage II	Carcinoma extending beyond cervix but not to pelvic sidewall; carcinoma involves vagina but not its lower third
Stage IIA	Involvement of upper two-thirds of vagina, no parametrial involvement
Stage IIB	Obvious parametrial involvement
Stage III	Carcinoma extending onto pelvic wall; on rectal examination, there is no cancer-free space between tumour and pelvic sidewall. The tumour involves lower third of the vagina. All patients with hydronephrosis or non-functioning kidney are included unless known to be the result of other causes.
Stage IIIA	Involvement of lower third of the vagina; no extension to pelvic sidewall
Stage IIIB	Extension to pelvic sidewall and/or hydronephrosis or functioning kidney
Stage IV	Carcinoma extends beyond true pelvis or clinically involves mucosa of bladder or rectum. Bullous oedema does not allow a case to be designated as stage IV.
Stage IVA	Spread of growth to adjacent organs
Stage IVB	Spread to distant organs

Source: Adapted from Waggoner (2003).

The lesion size in the cervix and its extension to adjacent regions guide clinical tumour staging (Waggoner, 2003). Findings on chest x-ray help to locate metastatic involvement in the lungs and distant lymph nodes. Ultrasonography images the kidneys and bladder for any obstruction by the tumour.

Although the current FIGO staging system does not recognise endometrial invasion as a criterion to staging cancer of the cervix, studies have shown its significance in predicting poor prognosis after irradiation (Perez *et al*., 1981).

Other factors that require consideration when determining the outcomes of cancer treatment are the size of the tumour, the involvement of the lymph nodes, the stage, cervical stromal invasion and lymphovascular space invasion (Miller & Grigsby, 2002). Furthermore, Grigsby and colleagues (2001) proved that FDG-PET-detection of lymph node metastases was more accurate at predicting the outcomes in cervical cancer than by the FIGO stage which is clinically based.

A review by Stephman and his colleagues (1991) earlier deduced that patient age and tumour stage were inferior in predicting the survival outcome and demonstrated that the size of the tumour and metastasis to the para-aortic lymph nodes were “the two most significant prognostic factors for survival outcome” (Singh *et al.*, 2002).

2.3 MANAGEMENT OF CERVICAL CANCER

2.3.1 Preventative Methods

The South African national guideline for cervical cancer screening programme (DoH, n.d.) has listed some preventative measures to help combat cervical cancer before it becomes difficult and too expensive to manage.

2.3.1.1 Primary Prevention

- “Stop smoking or preferably never start smoking. There is evidence that women who smoke are more susceptible to cervical cancer than women who do not smoke;
- Use barrier methods during intercourse to prevent the spread of the HPV and other sexually transmitted diseases;
- Postpone sexual activity to older age;

- Effectively manage sexually transmitted diseases; and
- Decrease parity." (DoH, n.d.).

2.3.1.2 Secondary Prevention

- "Cervical cytology (Pap) smear is proposed for the programme.
- Three free smears per lifetime are proposed.
- Women screened for the first time at age 55 or more will have only one smear if first smear is normal.
- A woman with an inadequate smear should be re-screened. If the second smear is also inadequate, the patient should immediately be referred to a known competent screening service.
- Women with any gynaecological complaints requiring a smear should receive a smear as part of the routine gynaecological examination. This is a diagnostic investigation and is not regarded as an element of the screening programme.
- Should more than three smears be requested by a woman, the extra cost will have to be carried by her." (DoH, n.d.).

2.3.2 Surgery, Chemotherapy and Radiotherapy

The management of cervical carcinoma is structured as follows:

2.3.2.1 Pre-cancerous lesions

Biopsy and histological studies confirm the type of lesion on the cervix. Lower grade SIL goes untreated and the patient is booked for re-examination after an appropriate period, whereas an HSIL is removed by a number of means further explained below.

2.3.2.2 Early stage cervical cancer (micro-invasive disease stage Ia to macroscopic disease confined to cervix <4cm, Ib1)

In stage Ia1 the removal of the lesion is performed through laser surgery which includes large loop excision of the transformation zone (LLETZ) or surgical conisation. Another frequently applied method is the use of cryosurgery to destroy the lesion. However, should the lesion recur, protocol strongly advises that the patient undergo a total hysterectomy (Denny, 2010).

Gershenson (2005) reported that a stage Ia2 and Ib1 cancer is often treated by radical hysterectomy which involves the removal of parametrial tissues (uterus, cervix, fallopian tubes and ovaries), the upper third of the vagina and the pelvic lymph nodes, however the patient's desire to remain fertile is considered.

Moreover, Gershenson (2005) suggested that radical trachelectomy is an option for patients who wish to preserve their fertility. In this surgical breakthrough, the uterus is spared while the cervix, parametrium, pelvic lymph nodes and the upper 2cm of the vagina are removed. The uterus is then carefully attached to the remnant of the vagina and "a cerclage is placed where the cervix used to be, to allow the patient to carry a pregnancy" (Molhere, 2008).

In the case of the presence of risk factors such as tumour size > 2 cm, deep cervical stromal invasion, involvement of pelvic nodes and parametrial invasion, adjuvant radiation and chemoradiation can be added (Peters *et al*., 2000; Plante, Renaud & Roy, 2005).

2.3.2.3 Locally advanced cervical cancer (Stage Ib2 to IVa)

A number of publications have illustrated the benefit of chemoradiation over radiation alone in locally advanced cervical cancer (Green, Kirwan & Tierney, 2001; Dueñas-Gonzalez *et al*., 2003). This evidence led to a consensus of standardising cisplatin-based chemoradiation and incorporating it as routine treatment for locally advanced cervical cancer patients (Candelaria *et al*., 2006).

The goal is now focused on increasing tumour sensitivity to radiotherapy. A monoclonal antibody, cetuximab, with cytotoxic radiosensitiser characteristics has found practical application as an adjunct to chemoradiation (Candelaria *et al*., 2006).

2.3.2.4 IVb and recurrent disease

Chemotherapy with or without radiotherapy is recommended in stage IVb and recurrent disease (Msibi, 2010).

Lymph node metastasis is almost always predictable in cervical cancer as it follows a pattern starting from the primary tumour in the cervix to the pelvic nodes, the para-aortic and supraclavicular lymph nodes then to the lungs, liver and bone (the distant metastatic sites) (Morice *et al*, 2000).

Previous studies have reported that survival rates diminish with increased nodal involvement. Examples include a 50% survival rate reduction in patients with positive pelvic lymph nodes as opposed to those who were not in the 1998 trial done by Comerci and colleagues. Grigsby and his team (2001) further reported a 30% survival rate in those with

para-aortic involvement leaving no hope for those with supraclavicular metastasis (Singh *et al*., 2002).

2.3.3 Role of PET/MRI/CT in evaluation of cervical cancer

The use of magnetic resonance imaging (MRI) and computed tomography (CT) has been advantageous to detect abnormal masses which provide information on the morphology to differentiate tumour lesions from normal structures (Welchalekar, Sharma & Cook, 2005). These diagnostic modalities achieve anatomical resolution, tissue differentiation and high imaging speed, lacking the identification of metabolic aspects of the tumour (Wechalekar, Sharma & Cook, 2005).

A cervical cancer study by Mayr *et al*. (1993) showed that MRI is particularly useful in adding information about the volume of the lesion and metastasis in terms of lymph node involvement. Subak *et al.* (1995) later concluded that "MRI offered significantly improved evaluation of tumour size, stromal invasion, location and regional extent of disease in pre-treatment imaging for cervical cancer" in comparison to CT. Although CT has been superseded by MRI, its application has been focussed onto staging advanced cervical cancer (>stage IIb) and in lymph node metastasis (Hricak & Yu, 1996).

Nuclear medicine employs PET and SPECT as its investigative tool. The target area and the perfusion to the target area are viewed from different planes. Blood flow images are planar images obtained immediately post-injection of a tracer. These are 2-5 second images that characterise perfusion to the lesion. Blood pool scans are usually obtained within 3-5 minutes post-injection; they visualize relative vascularity in the area of interest. Static scans are planar images obtained hours post-injection (1-24 hours) when renal clearance has decreased the tracer concentration in background tissue. These demonstrate the target to background turnover (Clifford, 2011).

Positron emission tomography (PET) on the other hand provides information on the functional or metabolic status of lesions by imaging glucose uptake in cancer cells (Grigsby, Dehdashti & Siegal, 1999; Rose, *et al*., 1999b; Reinhardt *et al*., 2001). It has been established that combining PET/CT produces optimum results with information on both the glucose utilisation [with respect to flouro-deoxyglucose (FDG) as a tracer] and the structure of the lesion (Beyer, Townsend & Blodgett, 2002; Grisaru *et al*., 2004; Kim, 2004). In addition, the image produced has improved localisation and specificity whilst minimising the weaknesses of each individual technique.

Single photon emission computed tomography (SPECT) as well as PET and CT employ tomographic techniques to reconstruct images of the target organ. The target organ is viewed in slices or focal planes over a 360º angle. Tomography makes it easier to visualise organs in 3D view, unlike planar techniques that only give 2D images (Saha, 2004).

SPECT is similar to PET in that it is predominantly the function of the organ that is visualised rather than just its morphology/structure. SPECT however differs from PET in that it uses gamma-emitting radionuclides which include ^{99m}Tc, ^{131}I and ^{67}Ga; whereas PET uses positron-emitting radionuclides (Theobald 2010).

The application of SPECT in cancer research, particularly tumour hypoxia, is widespread with the use of ^{99m}Tc as the radionuclide of choice to label the tracers (Yang *et al*., 1999; Chu *et al*., 2003; Song *et al*., 2003; Mallia *et al*., 2005; Giglio *et al*., 2011). Many advantageous properties of ^{99m}Tc previously described (Yang *et al*., 1999; Song *et al*., 2003) have led to its repeated selection, these are listed in detail in Section 2.3.5 below.

2.3.4 Complications

Hypoxia is a common trend amongst most solid tumour cells. The presence of hypoxia has caused many problems with respect to its management, regardless of whether it is radiotherapy (Gray *et al.*, 1953) or chemotherapy, for example cisplatin, which was shown to be resisted by some hypoxic stress proteins (Murphy *et al*., 1994).

Hypoxic conditions mean reduction in oxygen supply to cells leading to increased oxygen demand. Tumour cells have the ability to adapt to hypoxic conditions simply by inducing gene expression for hypoxia-inducible factor 1 (HIF-1). This substance attaches itself to vascular endothelial growth factor (VEGF) (Iyer, 1998) and erythropoietin (EPO) (Beck *et al*., 1991; Semenza, 1996; Pugh *et al*., 1997), ultimately promoting angiogenesis and erythropoiesis.

Studies have also shown that the binding of HIF-1 to glucose transporters and glycolytic enzymes (Iyer, 1998) leads to glycolysis (Semenza *et al.,* 1991), a cycle that needs no oxygen (anaerobic metabolism). Findings by Tanaka *et al*., (2005) also show that hypoxia stimulates VEGF expression resulting in increased vascularity of squamous cell carcinomas.

The use of oxygen electrodes (Eppendorf GmbH, Hamburg, Germany) to determine tumour hypoxia prior to surgical excision and radiotherapy brought a lot of hope for many, but its use later proved to negatively affect cervical cancer patients undergoing radiotherapy. There is documented evidence that this pre-treatment modality “was associated with pelvic failures

and with a decreased survival rate" (Fyles *et al*., 1998; Höckel *et al*., 1998; Dehdashti *et al*., 2003). Dehdashti *et al*. (2003) further identify invasiveness, technicality and the inability to access deep tumours as the major shortcomings of oxygen electrodes which has limited their routine use in clinical practice.

Non-invasive modalities have been presented as potential tumour markers for hypoxia such as PET with ^{18}FMISO (Rasey *et al*., 1990; Koh, Rasey & Evans, 1992; Overgaard & Horsman, 1996; Rasey *et al*., 1996; Fyles *et al*., 1998) and ^{60}Cu-ATSM-PET (Dehdashti *et al*., 2003). ^{18}FMISO showed a low clearance among other unfavourable characteristics listed below and although ^{64}Cu-ATSM showed a rapid clearance, high selectivity for hypoxic tissue and rapid wash out from oxygenated cells in *in vitro* studies by Lewis *et al*. (1999), it still is more expensive to produce because it requires a large cyclotron (restricted to large research institutions) and more energy to produce.

2.3.5 ^{99m}Tc-EC-MN: a tracer for hypoxia

The radiopharmaceutical ^{99m}Tc-EC-MN consists of metronidazole (MN) which is known for its antibacterial and antiprotozoal effects (AHFS, 2008). The radioisotope ^{99m}Tc is bound to the metronidazole via the chelating agent ethylene-dicysteine (EC). In a study by Nunn, Linder & Straus (1995) metronidazole, a nitroimidazole, was found to possess some of the ideal properties required to detect hypoxia in tissue. Metronidazole has the ability to be trapped in hypoxic cells and when administered linked to a radionuclide this results in high resolution of hypoxic-to-normoxic tissue ratio. At low oxygen tension metronidazole cannot be efficiently reoxidised, following reduction, and therefore subsequent reduction takes place. Further reduction retards its ability to diffuse out of the cell. The selectivity of metronidazole also stems from its binding affinity to tumour lesions and brain tissue (Song *et al*., 2003) and the myocardium (Nunn, Linder & Strauss, 1995).

A question of significance would then be, "How comparable is ^{99m}Tc-EC-MN to other markers of hypoxia?" Yang *et al*. (1999) assessed the feasibility of using ^{99m}Tc labelled metronidazole as a tumour hypoxia marker compared to existing markers such as ^{18}Fluorine-fluoromisonidazole (^{18}FMISO) and [^{131}I] iodomisonidazole (^{131}IMISO). ^{99m}Tc-EC-MN showed a significant increase in tumour-to-tissue uptake ratio in a shorter period post injection. Another positive point is that it showed metabolic stability unlike ^{131}IMISO which accumulated in thyroid tissue as well. The study findings proved thefeasibility of ^{99m}Tc-EC-MN to image tumours.

Song *et al*. (2003) preferred using ^{99m}Tc-EC-MN over ^{18}FMISO based on the attractive characteristics, i.e. easy accessibility and low cost of ^{99m}Tc in comparison to ^{18}F which is cyclotron produced and is therefore expensive with short half-life. ^{99m}Tc-EC-MN has been used to study acute ischaemia stroke with brain SPECT in patients who were recovering.

On conclusion Song *et al*. (2003) proved that ^{99m}Tc-EC-MN can predict the outcome of in ischaemic stroke. This evidence led to the choice of ^{99m}Tc-EC-MN as an affordable, effective and highly sensitive hypoxic marker for cervical tumours..

2.4 ^{99M}TC-EC-MN

2.4.1 ^{99}Mo/^{99m}Tc Generators

^{99}Mo/^{99m}Tc generators have simplified access to pertechnetate in that they are easily transported to the facility where it is used and supplies sufficient pertechnetate in a relatively quick and safe manner. The generator is constructed on the principle of the decay-growth relationship between a long-lived parent radionuclide (^{99}Mo: half-life=66 hours) and its short-lived daughter radionuclide (^{99m}Tc: half-life=6 hours). In order for separation to occur, the chemical properties of the daughter radionuclide must be different from those of the parent radionuclide. The generator contains ^{99}Mo in the form of molybdate (MoO^{-}_{4}) which is adsorbed onto alumina (Al_2O_3). Separation is achieved by elution with normal saline because the double negative charge on the molybdate ion causes it to adhere more strongly to alumina than ^{99m}Tc which has a single negative charge (TcO^{-}_{4}) (Knoesen, 2011).

2.4.1.1 Elution process

1. An empty sterile vial is cleaned by wiping the rubber plug with an alcohol swab.
2. The sterile vial is placed in a lead shield with a transparent graded scale with which to measure the amount of the eluate.
3. An empty safety steri-vial covering the needle is removed. The vial has positive pressure and therefore does not suck in sodium pertechnetate from the generator.
4. The lead-shielded sterile vial (steri-vial) is then placed into a protruding needle on the generator. This vial is like a vacuum and sucks in sodium pertechnetate from the generator.
5. Once the sodium pertechnetate is received and the vial is filled, the vial is then removed and the safety steri-vial is replaced to cover the needle.

6. Molybdenum breakthrough is then measured using a detector. The purpose of this action is to measure the amount of ^{99}Mo present in the solution thereby determining the purity of the pertechnetate (IAEA Nucleus, 2010).

2.4.2 Detection of ^{99m}Tc gamma rays

Gamma rays consist of photons that interact with orbiting electrons which are ejected and may interact with atoms to produce charged ions.^{99m}Tc emits gamma rays of 140KeV energy (Theobald 2011). Theobald (2011) further stated that detectors "function in two ways:

i. The radiation causes ionisation within a medium it is passing through and the ions produced are detected and measured.

ii. The radiation causes electronic excitation in atoms or molecules, which then dissipate this excess energy by some mechanism that can be detected and measured."

Scintillation detectors, semi-conductors and gas ionisation chambers are the main counting systems used in various analytical procedures in radiopharmacy.

Radionuclide Chromatogram Scanners are commonly used by most nuclear medicine departments to analyse the radiochemical purity of radiopharmaceuticals. The Veenstra chromatogram scanner (VCS-201) has a sodium iodide/thallium (NaI/TI) detector which is suitable for SPECT isotopes e.g. ^{99m}Tc. A low or high-energy collimator is connected to the detector to minimise scattered gamma rays. Collimator type selection is dependent on the energy of the emitted rays of the radioisotope used for a particular procedure. PET isotopes for example normally emit high-energy gamma rays whereas SPECT isotopes emit low-energy gamma rays. Analysis of the acquired data is through a multi-channel analyser card which contains the software that enables the system to perform data acquisition and analyse the detector signal (radioactivity) to produce a graphically illustrated chromatogram or spectrum that can be quantitatively interpreted (Veenstra Instruments, 2011).

2.4.3 Kinetics of Metronidazole

When given as an antiprotozoal, metronidazole reaches a peak concentration in 1-2 hours with a bioavailability that approaches 100%. It is widely distributed appearing in bile, bone, breast milk, cerebrospinal fluid, the liver, saliva, seminal fluid, vaginal secretions and erythrocytes (AHFS, 2008; Sweetman, 2002). Metronidazole is metabolised in the liver by hydroxylation, side-chain oxidation and glucuronide conjugation to its metabolites, namely,

1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (which has antibacterial activity) and 2-methyl-5-nitroimidazole-1-acetic acid (no antibacterial activity). Other metabolites are formed in the gut by normal flora (Koch *et al*., 1981). Metronidazole's main route of excretion is via the kidneys as metabolites and a small portion is also found in faeces and breast milk (Sweetman, 2002).

Metronidazole is unstable when exposed to light over time and it changes colour. Storage of injection formulations in a light-resistant container prevents decomposition associated with light. It is stable in air and must be stored between 15-30ºC and kept from freezing. A commercially available injection, RTU® that is manufactured by Baxter, has an expiry date of 24 months. Formulations that require reconstitution appear clear, pale yellow to yellow-green and have a very low pH of 0.5 to 2. Once reconstituted, they are chemically stable for only 96 hours in room light at less than 30ºC (AHFS, 2008).

2.4.4 Kinetics of EC-MN

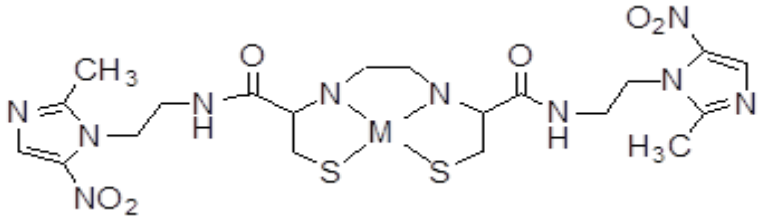


Figure 2.1: Structure of 99mTc-EC-MN (M: ^{99m}Tc=O)

Figure 2.1 shows the structure of ^{99m}Tc-EC-MN. ^{99m}Tc-EC-MN is the combination of ethylene-dicysteine, a chelating agent, and two molecules of metronidazole, the hypoxia tracer (Yang *et al*.,1999)

Yang *et al*. (1999) and Verbruggen *et al*. (1992) supported the use of ethylene dicysteine as a chelating agent for metronidazole because of its efficiency and ease when labelling at room temperature. In addition, it produced high radiochemical purity and the product remains stable for 8 hours.

2.4.5 Quality Control

The current good radiopharmacy practice guidelines (cGRPP) (Elsing *et al*., 2010) state that every radiopharmacy should have a quality assurance unit in place. Some of the responsibilities of the unit are to ensure that "radiopharmaceuticals are only supplied for use in patients if they have been correctly processed, checked and stored in accordance with the defined procedures and released by a competent person" and that "adequate measures are

in place to ensure that radiopharmaceuticals are released, stored and handled in such a way that the required quality can be assured throughout their shelf-life and in accordance with the in-use expiry date" (Elsing *et al*., 2010). It is therefore essential to test for the radiochemical purity of ^{99m}Tc-EC-MN.

The British Pharmacopoeia as cited by Theobald (2011) defines radiochemical purity "the ratio, expressed as a percentage of the radioactivity of the radionuclide concerned that is present in the radiopharmaceutical preparation in the stated chemical form, to the total radioactivity of that radiopharmacy present in the radiopharmaceutical preparation". When a radiopharmaceutical has a radiochemical purity that is below the set limit, it compromises its uptake in the target organ and increase the background resolution, which gives poor-quality images and exposes the patient to unnecessary radiation (Saha, 2004). The impurities often observed are mainly 'free' pertechnetate and other complexes (Theobald, 2011) arising from "decomposition, temperature and pH changes, light, radiolysis and the presence of oxidising or reducing agents" (Saha, 2004). It is when these impurities are above the acceptable quantities as set by the manufacturer that the purity of the radiopharmaceutical is compromised (Theobald, 2011).

2.4.6 Thin-layer Chromatography

Quality control (QC) procedures allow for the determination of impurities by various techniques. In the case of radiochemical purity testing, the most commonly practiced technique is planar chromatography. "Planar chromatography includes paper, thin-layer and high performance thin-layer chromatography. The techniques are similar in that a sample is applied to the stationary medium and, after equilibration in a closed vessel with the mobile phase, is then developed for an appropriate distance or time" (Theobald, 2011).

Thin-layer chromatography (TLC) was the chosen method for this study because of its popularity, ease of performance, economy (Theobald, 2011) and its speed, taking just minutes to perform (Saha, 2004). The selection of instant thin-layer chromatography (ITLC) was based on a similar study by Yang and his colleagues (1999) and its small scale rapid QC testing has rendered it suitable for routine application for daily QC in radiopharmacies which is widely practiced (UKRG Handbook, 2011)

TLC is governed by a few principles which state that the sample under analysis has to be in very small quantities (microgram quantities); the solvent has to move the sample across a flat surface; the components of the sample separate at different rates according to the solubility, adsorbability, size or charge. Elution is stopped when the solvent reaches the

opposite side of the chromatogram (solvent front). The separation process in TLC is mainly by surface adsorption where the sample components move along the stationary phase “at rates governed by their distribution ratios and therefore separate into individual spots or bands” (Fifield & Kealey, 1995).

2.4.6.1 Stationary phase

TCL usually uses a sheet of glass, plastic plate or aluminium foil that has a layer of silica gel, silica acid, alumina, and charcoal or cellulose powder as stationary phases (see Table 2.2 below). While ITLC uses glass fibre strips impregnated with silica gel or silica acid. These are also known as adsorbents that retard the molecules of the sample which is carried by a solvent flowing up the plate. The solvent movement is by capillary action (Fifield & Kealey, 1995).

Table 2.2: Stationary phases for thin-layer chromatography

Stationary Phase	Predominant Sorption Process	Use
Silica gel	Adsorption or partition	General
Modified silica gels	Adsorption or partition	Similar to bonded phase HPLC
Alumina	Adsorption or partition	General
Cellulose powder	Partition	Inorganic, amino acids, nucleotides, food-dyes
Kieselguhr	Partition	Sugars
Modified celluloses e.g.DEAE and CM	Ion exchange	Nucleotides, phospholipids
Sephadex gels	Exclusion	macromolecules

Adapted from Fifield & Kealey (1995)

The molecules which have a greater affinity for the adsorbent/stationary phase are retarded more than those which tend to have a weaker affinity. The molecules that bind strongly travel a shorter distance than those which are weakly bound to the stationary phase. This is how the retardation factor (Rf) (Fifield & Kealey, 1995) values are determined, details in section 2.4.2.3 below.

2.4.6.2 Mobile phase

The polarity of a solvent greatly impacts its ability to carry components of a mixture along the stationary phase (Saha, 2004). An important property that a solvent should have is its selectivity for substances and its ability to dissolve and separate the sample into its different constituents. A substance that is soluble in a solvent is eluted faster. Another important factor is the solvent’s adsorbability to the adsorbent. The ideal solvent must be more strongly

adsorbed than some components and less strongly adsorbed than others. This will speed up the elution rate of those components that show a lower adsorption to the stationary phase than the mobile phase. The components that are more strongly bound to the stationary phase will be affected less or not at all (Theobald, 2011).

Table 2.3 below lists common solvents used in chromatography. Water has a strong eluting power as it is highly adsorbed; therefore solvents used in chromatography must not contain water unless a combination has been deliberately used with 0.5 - 2% v/v addition.

Table 2.3: Eluting solvents for chromatography in order of increasing adsorbability

Least eluting power (silica as adsorbent)	n-Pentane
	Hexane
	Cyclohexane
	Carbon tetrachloride
	Toluene
	Chloroform
	Methylene chloride
	Tetrahydrofuran
	Acetone
	Ethyl acetate
	Aniline
	Acetonitrile
	Ethanol
	Methanol
	Water/Saline
Greatest eluting power (silica as adsorbent)	Acetic acid

Partly sourced from: Monzir, 2011

2.4.6.3 Retardation Factor

Saha (2004) defines the retardation factor (Rf) value as ''the ratio of the distance travelled by the component to the distance the solvent front has advanced from the original point of application of the test material''. Mathematically, it is notated as:

$$Rf = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}} \qquad \text{Eq2.1}$$

The Rf value of a solute is determined by its distribution ratio, which in turn is dependent on relative solubilities for partition systems or relative polarities for adsorption systems (Fifield & Kealey, 1995). Therefore, "during the chromatographic process, different components of the sample distribute themselves between the adsorbent and the solvent, depending on their distribution coefficients" (Saha, 2004). A polar compound will have a low Rf value if it travels on a polar adsorbent (stationary phase) as it is retarded, resulting in a shorter distance travelled, provided the solvent is non-polar or is less polar than the compound. A non-polar

compound on a polar adsorbent will be carried over a longer distance displaying a larger Rf value, depending on the properties of the solvent, as well (Saha, 2004; Denthre College, 2010). “The properties of the solvent are also critical” (Knoesen 2011). For example, if a strong polar solvent is used to elute a polar compound, the compound will be carried to the solvent front.

Factors affecting Rf values are summarised below (Fifield & Kealey, 1995):

- Temperature: high temperature catalyses the separation but lowers the resolution due to increased rates of diffusion. Samples and standards must be run simultaneously to allow for comparison.
- Quality of thin layer material: the plate should have uniform thickness ranging from 0.2 - 0.3 mm to prevent variations in Rf value.
- Atmosphere: a stable, saturated atmosphere within the developing tube is required to ensure that Rf values are reproducible. A plug is often used to prevent evaporation of the solvent (which would increase solvent flow but slow movement of the solvent front, ultimately increasing Rf values).
- Sample size (spot): large samples alter Rf values and resolution significantly. The optimum sample size is 0.01 – 50 µl.

Another method of expressing radiochemical purity is through ‘normalisation’ in which the radiochemical yield is expressed in percentages calculated using the following formula (Theobald, 2011):

$$\text{Percent component} = \frac{\text{Radioactivity of component}}{\text{Total radioactivity}} \times 100 \qquad \text{Eq 2.2}$$

2.4.7 Tin (II) chloride solubility

Tin (II) chloride, also known as stannous chloride, is a well-known and widely used compound. In radiopharmacy it has found application as a reducing agent in the preparation of radiopharmaceuticals, particularly those containing pertechnetate. Technetium exists in eight oxidation states of which only two, namely, +7 and +4 are naturally stable and which are present in pertechnetate and technetium colloid (reduced hydrolysed technetium/ technetium dioxide). The eluted form of technetium from a ^{99}Mo/^{99m}Tc generator is pertechnetate, which is used for radiolabelling (Saha, 2004).

The labelling efficiency is analysed with chromatography, where free pertechnetate, along with the labelled product, migrate with the solvent front. However, unbound ^{99m}Tc-colloid often remains at the origin in most TLC systems, irrespective of which mobile or stationary phase is used because insoluble material does not migrate (Zolle, 2007) (see Table 2.4).

Table 2.4: Expected Rf values of pertechnetate and ^{99m}Tc-colloid with ITLC-SG chromatography

Mobile phase	Pertechnetate	^{99m}Tc-colloid
Ethyl acetate	0.0	0.0
Acetone	0.6-0.1	0.0
Ethanol	0.6-0.1	0.0
Saline	0.6-0.1	0.0

Source: Zolle, 2007; Krcal *et al*., 2002

Unless technetium is reduced from the +7 state it cannot bind to a ligand. This is where tin (II) chloride ($SnCl_2$) comes into play as it acts as a reducing agent for pertechnetate (Saha, 2004).

Literature emphasises the difficulty in dissolving tin (II) chloride in water as it is immediately hydrolysed, forming insoluble compounds of tin. Not only does this phenomenon affect the labelling efficiency but it also poses a danger to the patient as there may be high quantities of free pertechnetate which will result in the undesired uptake in non-target organs (Saha, 2004).

2.5 SUMMARY

This chapter dealt with cervical cancer as a disease and its management. The complications of the disease, with tumour hypoxia as the prime focus, were addressed. Nuclear Medicine and radiopharmaceutical solutions with PET and SPECT were identified as solutions to hypoxia in early stage cervical cancer. The radiopharmacy aspects covered in the chapter included QC, chromatography and the equipment used in detection of gamma rays. The next chapter will give detail on the development of the method to assess labelling efficiency.

CHAPTER 3
METHOD DEVELOPMENT FOR PREPARATION AND QUALITY CONTROL OF ^{99m}Tc-EC-MN

3.1 INTRODUCTION

An existing protocol for the study of ^{99m}Tc-EC-MN had been approved by University of Pretoria Research and Ethics Committee (UPREC) (2006) (see Appendix 1). The protocol was edited and adapted by the researcher for the purpose of her MSc and submitted to Medunsa Research and Ethics Committee (MREC) for approval, which was received on the 26th November, 2008 (see Appendix 2).

There were six vials of EC-MN "in stock" at the University of Pretoria (UP) at the time of submission of the original protocol. Logistical delays between the involved departments at the University of Pretoria/Steve Biko Academic Hospital (UP/SBAH) meant that the project only began in November 2010.

The delays meant that the product, EC-MN, had been held at SBAH under refrigeration and in sealed amber vials for more than two years. There was no expiry date to which the investigator could refer. Efforts were made to obtain newly-produced product from the USA group where it was originally prepared, but it was found that the product was now under further commercial development and therefore not available. The new commercial product was not yet registered with the Food and Drug Administration (FDA). This meant that the options were either to abort the study or determine the stability and labelling efficiency of the available vials. In addition there was old stock of an earlier batch of EC-MN, which did have an expiry date (2007). This product was well past the expiry date and was contained in non-airtight Eppindorf tubes and held under refrigeration. It was decided that the expired product could be used for chromatographic comparison with the more recent batch of vials. **Hence the focus of the study changed from a purely clinical one to a more chemistry-based one.**

Concerns over whether the sealed vials of EC-MN were still suitable for use, lead to the decision to perform extensive quality control tests, before its administration to patients.

It is important to know the labelling efficiency as that has an effect on the bio-distribution of the radioactive products within the body. Pertechnetate (TcO^-_4) accumulation is visualised in the salivary glands, thyroid, GIT and the bladder. As detailed in the previous chapter

(Section 2.4.5), metronidazole is usually distributed mainly to the liver and the intestines, where cytochrome P450 enzymes are dominant. The product ^{99m}Tc-EC-MN is a radiopharmaceutical containing a ligand and a radioisotope. It is theoretically assumed that if labelling is correct or efficient, the distribution pattern of the ligand will dominate; therefore, ^{99m}Tc-EC-MN is expected to follow the normal distribution of metronidazole which also has been proven to accumulate in hypoxic tissue.

Similar analytical methods to those used by the product developer were followed as far as possible, Instant thin layer chromatography was the analytical method chosen to determine the stability and chemical constitution of the product. The developer used an unspecified ITLC-SG stationary phase plate, and a different radio-scanner (Bioscan, Washington DC), all correspondence failed, therefore it was decided that ITLC-SG (Pall Corporation, Pall Life Sciences) would be used and the available Veenstra chromatogram scanner (VCS 201) was used.

3.2 STUDY SITE

The study was conducted at Steve Biko Academic Hospital in the department of Nuclear Medicine. The radiopharmaceutical quality assurance studies were conducted within the “hot” laboratory and the SPECT images within the imaging rooms.

3.3 STUDY MATERIALS AND EQUIPMENT

The materials and equipment in (Table 3.1 below) were used during the method development. The QC process was conducted in the “hot” laboratory of SBAH and the Laboratories of NTP Radioisotopes. Scintigraphy was performed at the Nuclear Medicine Department at SBAH (see Chapter 4).

Table 3.1: Study materials

Materials
70% ethanol
Acetone
0.9 % NaCl / normal saline
Ethyl acetate
ITLC-silica gel paper
F254 silica gel plates
Sterile water for injection
Tin (II) Chloride / Stannous chloride
Technetium pertechnetate
1ml syringe
10ml syringe
10ml vials
Needles
Paper towels
Pencil
Ruler
Scissors
Micro-pipette
Test tubes and stoppers
Test tube stand
0.22µm filters (Millex-GV)
Laboratory coat
Film badge
Latex gloves
HCL 1M
NaOH 0.1M
NaOH 1M
Colour-coded pH strips HPLC grade acetonitrile (Merck)
1% tri-ethylamine (TEA)
Equipment
Geiger counter
$^{99}Mo/^{99m}Tc$ generator (Peltec)
Chromatogram scanner (Veenstra Instruments VSC-201)
Laminar air flow cabinet
Radio-HPLC Agilent 1200 Series Model coupled with a UV-Vis Diode Array Detector (G1315D)

3.4 METHOD

3.4.1 ^{99m}Tc-EC-MN preparation

The following instructions were obtained with the EC-MN vials from the developer (Yang *et al*., 1999).

“Each vial contains 5 mg of EC-MN. Each vial is for 1 patient .The labelling method is as follows:

1. Weigh Tin (II) Chloride ($SnCl_2$) 10 mg, and dissolve in 9ml sterile water (Not supplied by the developer.) .Make sure solution is clear.
2. Dissolve EC-MN in sterile water (0.3 ml).
3. Add 0.1 ml of tin chloride solution (from step-1).
4. Add pertechnetate (30 mCi) and q.s. to 1 ml with sterile water.
5. Draw the sample from the vial and filter through 0.22 µm filter into a sterile bottle.
6. Draw the dose (20-25 mCi) per patient.”

3.4.2 Chronological sequence of events

Table 3.2 summarises the chronological sequence of events for the quality assurance method development. Each step (with results where relevant) is described in detail in the sections as listed in the final column of this table.

Table 3.2: Chronological sequence of events

Action	Reason	See Section
1.Solubilisation of tin	The tin (II) chloride must be solubilised in order to reduce $^{99m}TcO^{-}_{4}$ to $^{99m}TcO^{3-}$ (+5 oxidation state).	3.4.3
2.Radiolabelling EC-MN	To form a radioactive tracer	3.4.4
3.^{99m}Tc-EC-MN chromatography I	The stability of the EC-MN was not known. Stability affects labelling efficiency. Hence the radiochemical purity of the radiolabelled product had to be established.	3.4.5
4.Radiolabelling detection with the ionisation chamber	To measure the activity at the origin and solvent front and calculate the labelling yield thereafter.	3.4.6
5.^{99m}Tc-colloid chromatography	To determine the efficiency of pertechnetate reduction by tin (II) chloride by reducing a pertechnetate solution in the absence of EC-MN, thereby determining the resultant ^{99m}Tc-colloid and free pertechnetate as an indication of efficiency of the reduction reaction	3.4.7
6.^{99m}Tc-EC-MN chromatography II	Compare the Rf value of ^{99m}Tc-EC-MN with pertechnetate and ^{99m}Tc-colloid thereby determining whether EC-MN had been successfully radiolabelled with pertechnetate	3.4.8
7. Pertechnetate chromatography I	The RF value of ^{99m}Tc-EC-MN was unknown. It was therefore necessary to determine the Rf for free pertechnetate, a by-product of poor labelling and Rf of ^{99m}Tc-EC-MN. The results were then used as a standard for comparison.	3.4.9
8.Pertechnetate chromatography II	To determine whether the size of the chromatography “spots” or drops technique affected the shape, size or position of the peaks of the scans.	3.4.10
9. Detection of labelling efficiency and stability of expired EC-MN	For future comparison of stability and labelling efficiency with the non-expired batch	3.4.11
10. Detection of labelling efficiency of vials of EC-MN	Comparison of stability and labelling efficiency with the expired batch	3.4.12
11.Labelling EC-MN for patient dose	To use as a tracer for hypoxic cervical cancer cells	3.4.13 & 3.4.14

3.4.3 Solubilisation of tin (II) chloride

Introduction 1

It is well documented that tin (II) chloride ($SnCl_2$) is hydrolysed in water (Kneen, Rogers & Simpson, 1971; Saha, 2004). Hence there were concerns over the practical feasibility of Step 1 of the process as tabulated above in Table 3.2. Tin (II) chloride forms insoluble compounds, such as chlorohydroxo tin (II) when mixed with water in which oxygen is dissolved. An example of the reaction that takes place is depicted in Eq 3.1 and 3.2 below. Hence argon must be bubbled in the sterile water for 20 min to remove oxygen. Argon is heavier than oxygen. After displacing oxygen from water it acts as a seal on the water to prevent re-penetration of oxygen (Knoesen, 2011).

$6\ SnCl_2\ (aq) + O_2\ (g) + 2\ H_2O\ (l) \rightarrow 2\ SnCl_4\ (aq) + 4\ Sn\ (OH)\ Cl\ (s)$ Eq3.1
(Nechamkin, 1968)

$SnCl_2 + H_2O \rightarrow Sn\ (OH)\ Cl\ (s) + HCl$ Eq3.2
(Knoesen, 2011)

Note: (s) = insoluble solid

(aq)= water soluble

Stannous chloride will however dissolve in an acidic medium (Saha, 2004).

Method 1

Ten mg tin (II) chloride was weighed in a 10 ml vial and supplied as such. Initially, approximately 1 ml sterile water which had been bubbled with argon gas was injected into the vial containing tin (II) chloride and slowly filled to 9 ml to dissolve it.

Results 1

The tin (II) chloride did not dissolve and insoluble compounds of tin (II) were formed.

Conclusion 1

Ten mg tin (II) chloride forms insoluble compounds of tin (II) (precipitate) with a milky solution when dissolved slowly in 1-9 ml of argon-bubbled water.

Introduction 2

Tin (II) chloride does not dissolve in water but is soluble in an acidic pH (Saha, 2004). However, a very low pH would be too acidic for IV administration and may also affect the stability of the EC-MN. Hence a buffer was considered. The pH was adjusted with sodium hydroxide. This procedure was performed using deoxygenated solutions under an argon atmosphere. A calibrated Metrohm Model 691 pH meter equipped with a standard pH probe was used to measure the pH.

Method 2

Tin (II) chloride was dissolved in 0.1ml, concentrated hydrochloric acid (HCl) and 9 ml sterile water was added while stirring, giving an approximate HCl concentration of 1 M. (Saha, 2004) The pH adjustment was performed with the drop wise addition of sodium hydroxide NaOH. . A 1 M NaOH solution was used to initially adjust the pH to approximately 4.5 Thereafter 0.1 M NaOH was used to adjust to pH 7. This is not a very good procedure as overshooting occurred frequently and was not reliable. Therefore the solubilisation in water was pursued.

Result 2

Tin (II) chloride dissolved in HCl, a clear solution was observed but the pH was too low (pH 2). When the pH was adjusted upwards, the tin precipitated from the solution before the required neutral pH was reached.

Conclusion 2

The approach of dissolving tin (II) chloride in HCl with subsequent buffering still produced a precipitate; therefore this approach is not suitable.

Introduction 3

The pH of the tin (II) chloride solution obtained in Method 2 above was very low. This would in turn lower the pH of the product (^{99m}Tc-EC-MN) which would have made it unsuitable for intravenous administration to a patient.

Method 3

It was therefore decided that the water dissolution in Step 1 of Section 3.4.1 above would be re-attempted, by adding 9 ml of water all at once as in the given method (Yang *et al*., 1999).

Result 3

After many unsuccessful attempts in which a precipitate was formed, 9 ml water in a 10 ml syringe was quickly injected and the vial was swiftly swirled simultaneously. This approach resulted in the dissolution of tin (II) chloride and a clear solution was observed.

Conclusion 3

Despite literature evidence to the contrary, the method as provided by Yang and co-workers did work, provided all the water (9 ml) was added rapidly to the 10 mg tin (II) chloride in one aliquot and shaken vigorously to produce a clear solution of tin (II) chloride. It was not deemed advisable to add tin (II) chloride to the water as 10mg is such a small amount that it would have adhered to the walls of the vial in which it was weighed, producing an inaccurate addition amount.

3.4.4 Radiolabelling EC-MN

Method

EC-MN was dissolved in 0.3 ml sterile water, and then 0.1 ml of the tin (II) chloride solution was added to it. Thirty mCi of the eluted pertechnetate was then added to the above solution and made up to 1ml with sterile water. The solution was then filtered through a sterile Millex-GV 0.22 µm filter (Millipore Corp Co. Cork, Ireland) with a hold up volume less than 0.1ml into a sterile vial.

Results

EC-MN was labelled with 30 mCi of pertechnetate and a pale straw-coloured solution was observed.

Conclusion

A clear yellowish solution was produced. Radiolabelling efficiency then had to be assessed.

3.4.5 ^{99m}Tc-EC-MN chromatography I

Introduction

The purpose of chromatography was to assess whether EC-MN was indeed labelled as purposed in the method above (Section 3.4.4). The TLC method that was supplied with EC-MN was unclear in some places. It was not possible to obtain clarity from the developers of EC-MN despite numerous attempts to contact them. It was therefore decided to select at least four commonly used solvent systems of varying polarity that would be compared, based on their ability to separate. The system that best separated the labelled EC-MN from its impurities would then be used for routine QC of ^{99m}Tc-EC-MN for further application in this study.

Preparation of chromatograph strips

Method

Strips of approximately 1 cm width were cut from sheets of chromatography paper, ITLC-SG (Pall Corporation, Pall Life Sciences) and SG F254 (Merck). Each strip was 100 mm in length; 10 mm lines were marked from each end of the strips and labelled as “origin” and “solvent front” respectively.

ITLC chromatogram scanner method

The ITLC-SG and SG F254 strips were the stationary phases used in the determination of the radiochemical purity of ^{99m}Tc-EC-MN. Four different mobile phases were used in the combinations listed in Table 3.3 below:

Table 3.3: List of mobile phases with the corresponding stationary phases

Mobile phase	Stationary phase	
	ITLC-SG	SG F254
Acetone	√	
Ethyl acetate	√	
Ethanol	√	√
Saline	√	

Small quantities (approx. 1 ml) of each mobile phase had initially been poured into each test tube and allowed approx. 3 min to saturate the atmosphere within each tube. A spot of filtered ^{99m}Tc-EC-MN was placed on the origin using a 1 ml syringe and gauge needle. The strips run in saline were not dried but the others were allowed to dry in air for a few minutes before development in test tubes with the listed solvents (Table 3.3). After development in the respective mobile phase each strip was dried and placed onto the chromatogram scanner (VCS-201, Veenstra, Netherlands) to quantify the activity and hence calculate the radiochemical purity.

Results

Acetone

^{99m}Tc-EC-MN (Figure 3.1) below showed a broad peak with a shoulder at the solvent front with a lot of residual activity on the chromatogram. It was impossible to determine whether the activity at the origin was from ^{99m}Tc-colloid or residual activity remaining on the chromatogram. Neither was it possible to distinguish ^{99m}Tc-EC-MN from pertechnetate at the solvent front.

Background activity was a scan area where there were no peaks, it differs from residual activity (activity remaining after the compound has migrated past a certain point but where a certain percentage of the activity remains behind). The residual activity could be due to a combination of the properties of the mobile &/or stationary phase, product breakdown or product modification and non-optimal separation conditions.

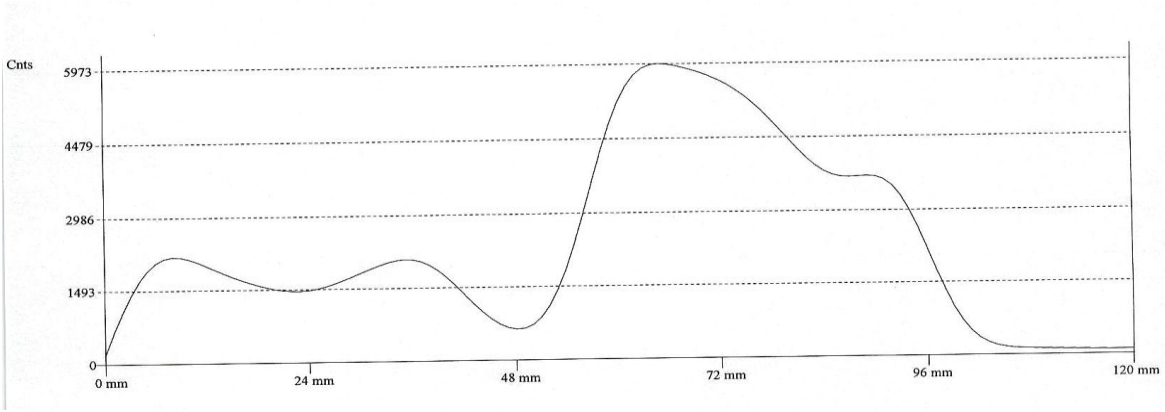


Figure 3.1: Chromatogram of ^{99m}Tc-EC-MN developed in acetone

Ethyl acetate

Figure 3.2 had a large peak at the origin, which is evidence of the presence of reduced hydrolysed technetium ($^{99m}TcO_2$) or ^{99m}Tc-colloid. There was also some residual activity and a smaller peak which did not separate well at the solvent front (possibly pertechnetate). However, a comparison with the ethanol and saline chromatograms (Figure 3.3 & 3.4, respectively) revealed that no ^{99m}Tc-colloid was present. Therefore the peak at the origin signifies ^{99m}Tc-EC-MN or free pertechnetate. The residual activity on the chromatogram was unacceptably high.

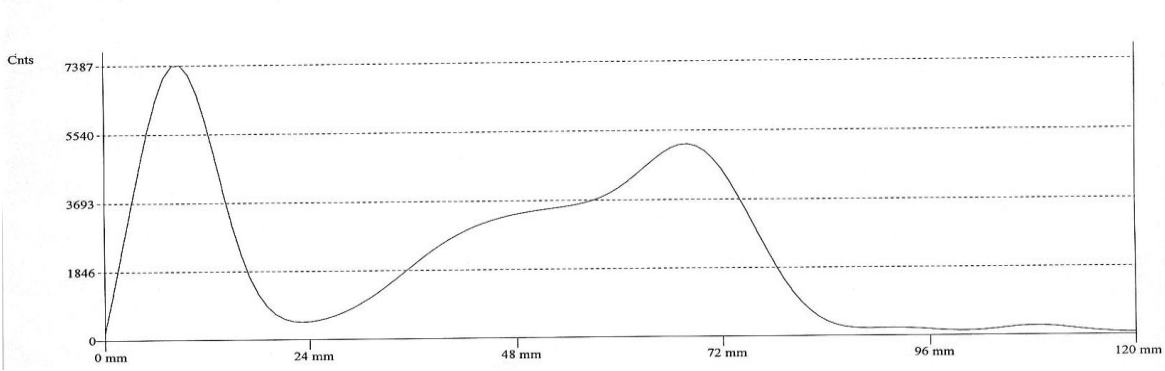


Figure 3.2: Chromatogram of ^{99m}Tc-EC-MN developed in ethyl acetate

Ethanol

^{99m}Tc-EC-MN developed in ethanol showed a broad peak with a shoulder. An approximate Rf value of 0.8 was calculated (Figure 3.3). No ^{99m}Tc-colloid was observed at the origin. Residual activity remaining on the chromatogram was less than the previous two chromatograms (Figure 3.1 & 3.2) but still evident.

The ethanol scan on the F254 plate as a stationary phase was too long to develop and was excluded from the experiments.

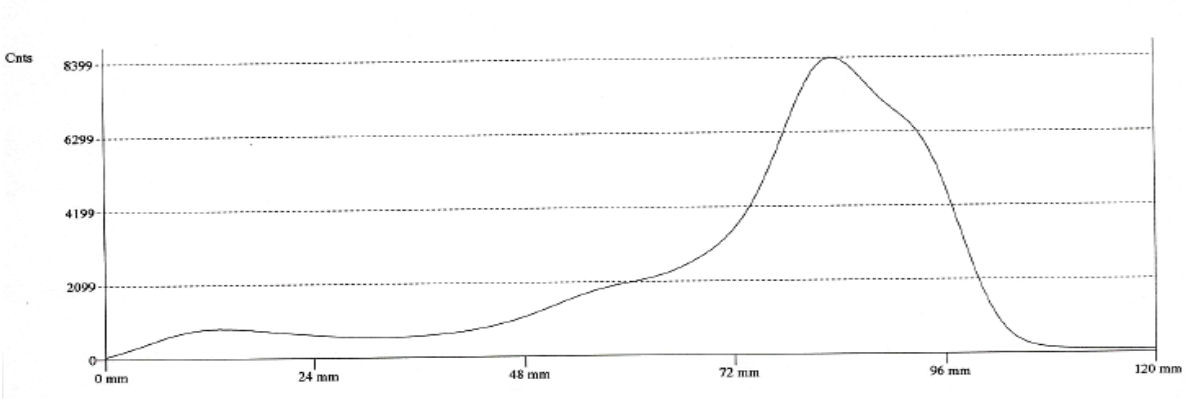


Figure 3.3: Chromatogram of ^{99m}Tc-EC-MN developed in ethanol

Saline

The saline mobile phase results below shows a clean peak (Rf=0.9) with little residual activity (Figure 3.4). No ^{99m}Tc-colloid was observed at the origin.

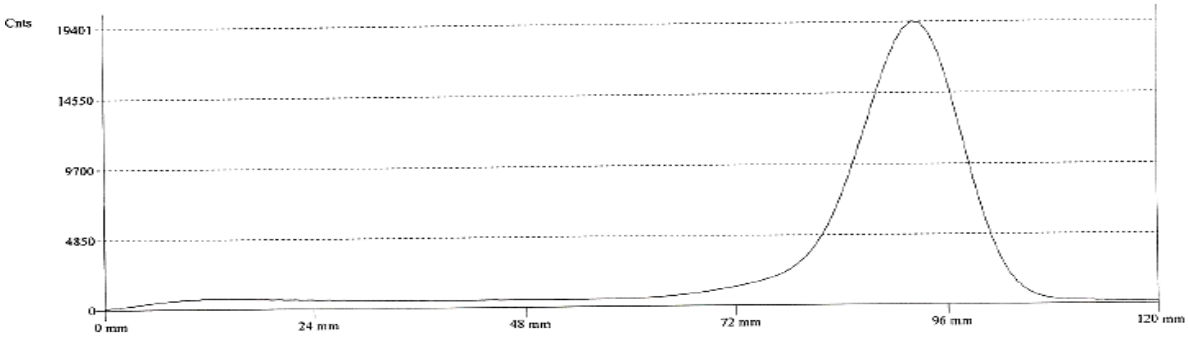


Figure 3.4: Chromatogram of ^{99m}Tc-EC-MN developed in saline

Conclusion

The acetone and ethyl acetate scans had multiple peaks and were poorly defined. These revealed features of poor separation, disqualifying the use of the two solvents for chromatography in this study. Ethanol and saline however showed better separation, in that no multiple peaks were observed. There was one peak at the solvent front. At that stage it was difficult to confirm that the identified peak signified labelling of EC-MN with pertechnetate. A method to differentiate the ^{99m}Tc-EC-MN peak and the peaks of its impurities (^{99m}Tc-colloid and pertechnetate) was therefore developed.

3.4.6 Radiolabelling detection with ionisation chamber

Method

ITLC was performed with the same combinations of solvents and stationary phases as listed in Table 3.3 above. Background activity within the ionisation chamber was measured first before radioactivity was measured on the strips. Each strip was initially cut in half; the origin and the solvent front halves were measured separately.

Results

Table 3.4: Measured activity of ^{99m}Tc-EC-MN chromatography strips

	Solvent	Activity at origin (µCi)	Activity at solvent front (µCi)	Percentage labelling yield
ITLC-SG	Saline	11	73	86.9 %
	Ethanol	18	53	74.6 %
	Ethyl acetate	66	35	34.7 %
F254 SG	Ethanol	83	7	8.4 %

The measurements in the above table (Table 3.4) showed that the required specification of 95% labelling yield was not achieved. The highest labelling yield was shown with saline as the mobile phase.

Conclusion

These measurements were not conclusive as the residual activity remaining on the chromatograph distorted the results.

3.4.7 ^{99m}Tc-colloid Chromatography

Introduction

^{99m}Tc-colloids may form as a by-product of inefficient labelling, giving a different Rf value from the sample under investigation. This experiment was performed to assess the efficiency of the tin (II) chloride solution in reducing pertechnetate quantitatively in the absence of EC-MN as a tool to assess labelling efficiency. The location of the reduced form of pertechnetate is always at the origin. Should tin (II) chloride reduction of pertechnetate not be efficient, a pertechnetate peak will be observed at the solvent front. If the pertechnetate peak is absent, then there is enough tin (II) chloride for reduction. Hence, if tin (II) chloride reduction has been sufficient then the peaks at the front of the chromatograms are most probably not pertechnetate.

Method

Tin (II) chloride solution was prepared as described in Section 3.4.4 and labelled with sodium pertechnetate to form ^{99m}Tc-colloid. Spotting of the ITLC-SG strips was performed in a similar manner as the ^{99m}Tc-EC-MN above (see Section 3.4.5). The radiochemical purity was measured on each ITLC-SG strip with normal saline and ethanol as the mobile phases.

Results

Ethanol

A distinct peak of ^{99m}Tc-colloid remained on the origin and no free pertechnetate was observed at the solvent front (Figure 3.5). This result is consistent with standards published in the British Pharmacopeia (BP) as stated in “Sampson’s Textbook of Radiopharmacy” (Theobald, 2011).

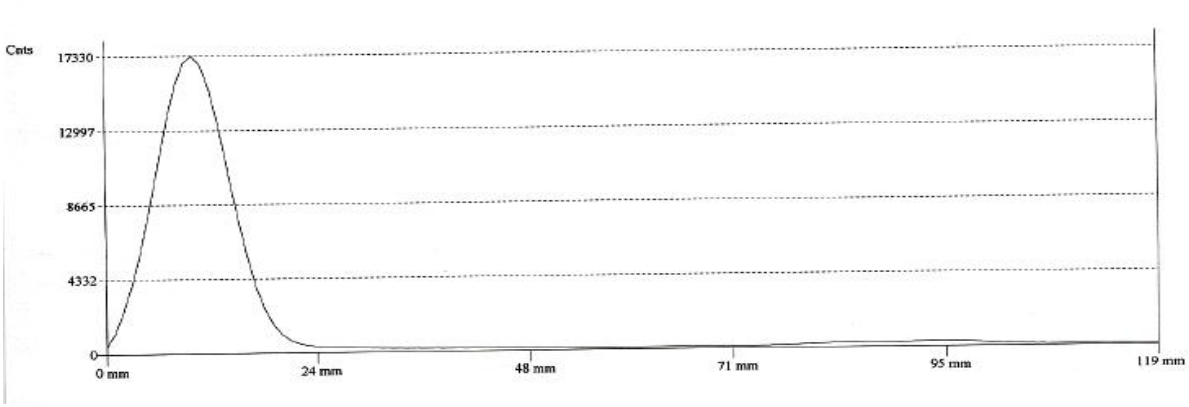


Figure 3.5: Chromatogram of ^{99m}Tc-colloid developed in ethanol

Figure 3.6, shown below, displayed a similar peak to the one observed above.

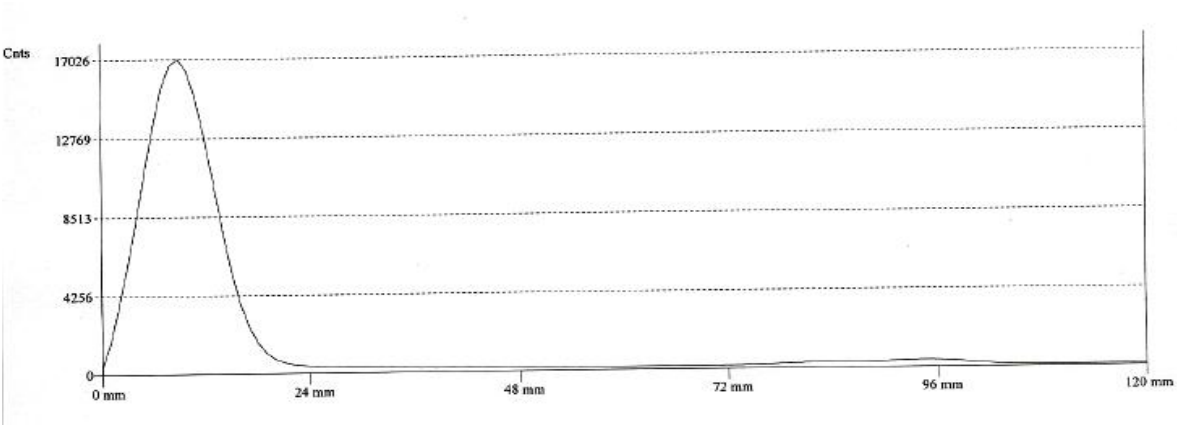


Figure 3.6: Chromatogram of ^{99m}Tc-colloid developed in saline

Conclusion

The pertechnetate solution was quantitatively reduced to ^{99m}Tc-colloid.^{99m}Tc-colloid remained at the origin (Rf=0.0) when it was developed in the polar solvents ethanol and saline.

3.4.8 ^{99m}Tc-EC-MN Chromatography II

Introduction

The saline mobile phase scan in Figure 3.4 of Section 3.4.5 showed a peak with an Rf value similar to that of documented pertechnetate scans. So it was unclear as to whether:

- the pertechnetate peak (the impurity) and the ^{99m}Tc-EC-MN were on the same Rf, thereby overlapping;
- there was no ^{99m}Tc labelling of the EC-MN at all or; and
- EC-MN was in fact successfully radiolabelled.

It was therefore necessary to repeat the labelled EC-MN chromatography in order to determine its peak and see if there might be identifiable separation.

Method

^{99m}Tc-EC-MN was prepared and chromatography performed as described above (see Section 3.4.5). The results were compared with those of ^{99m}Tc-colloid.

Results

Acetone

There was no difference between the peak in Figure 3.7 below and that of Figure 3.1 which was analysed in Section 3.4.5 above. The relatively high residual activity and multiple peaks were constant with the acetone-developed scans.

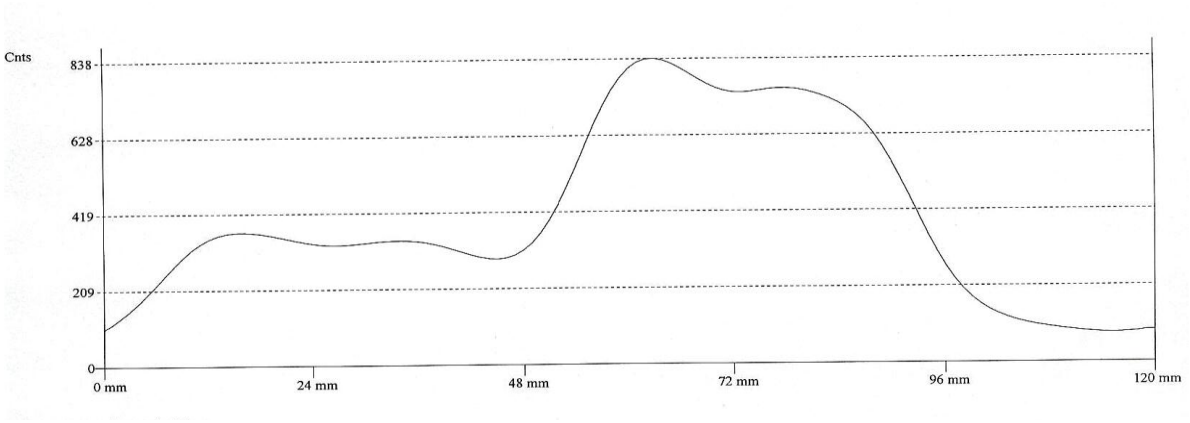


Figure 3.7: Chromatogram of ^{99m}Tc-EC-MN developed in acetone

Ethyl acetate

The peak in Figure 3.8 below also shows a lot of residual activity. The results were similar to the ethyl acetate-developed chromatogram performed the previous day (Figure 3.2). The peak at the origin was not ^{99m}Tc-colloid and ^{99m}Tc-EC-MN was suspected. Further analysis of ethyl acetate developed pertechnetate would clarify whether the peak was indeed ^{99m}Tc-EC-MN.

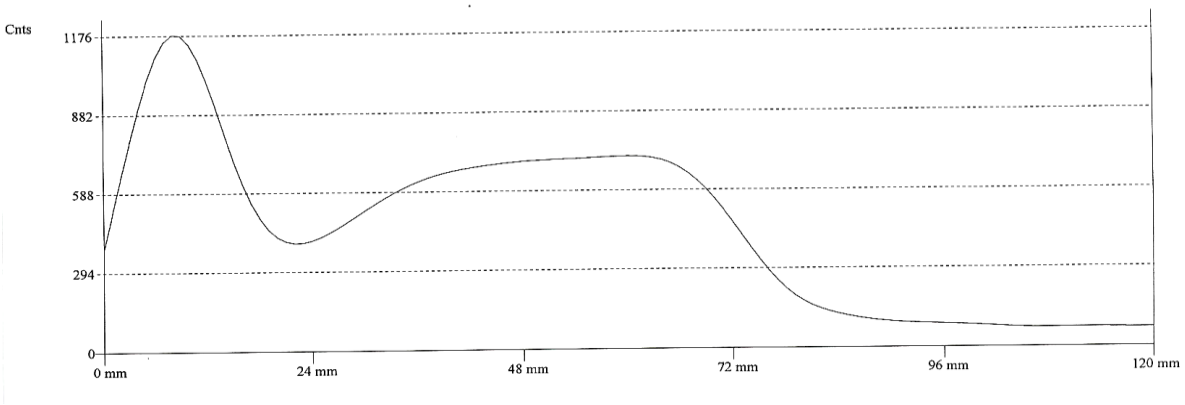


Figure 3.8: Chromatogram of ^{99m}Tc-EC-MN developed in ethyl acetate

Ethanol

Relative low residual activity was observed in Figure 3.9 shown below. Although the calculated Rf value of 0.8 remained unchanged, it had a better defined peak than that from the ethanol chromatogram shown in Section 3.4.5.

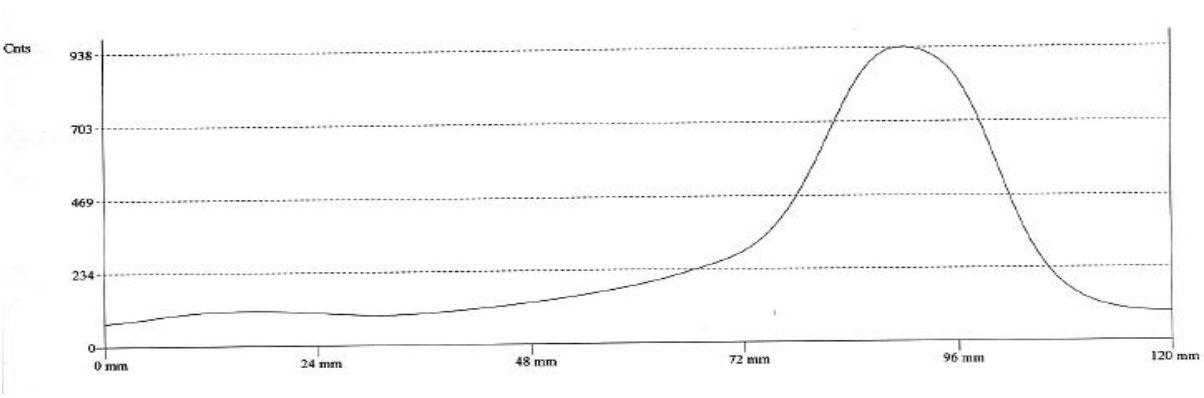


Figure 3.9: Chromatogram of ^{99m}Tc-EC-MN developed in ethanol

Saline

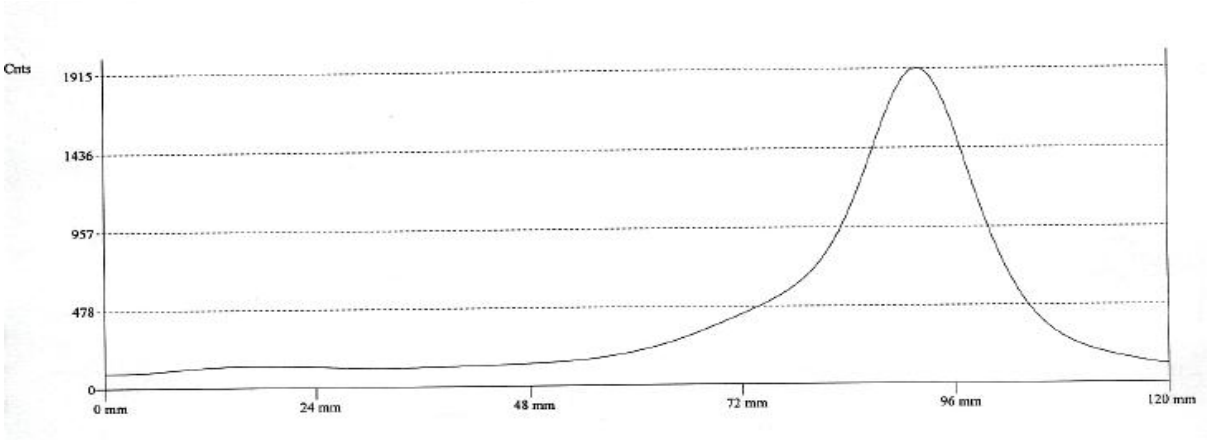


Figure 3.10: Chromatogram of ^{99m}Tc-EC-MN developed in saline

Figure 3.10 above showed an Rf value of 0.9. There was little residual activity. In comparison to the saline chromatogram in Figure 3.2 there is not much difference except that the peak in Figure 3.10 had a broader base (possibly) caused by photon scatter due to the thin collimator which is part of the Veenstra VCS 201 instrumentation design.

Conclusion

The Rf value of ^{99m}Tc-EC-MN appeared very close to that of pertechnetate in literature (Rf=1), (Theobald, 2011), when it was developed in ethanol and saline. The acetone continued to give poorly separated peaks as demonstrated in Section 3.4.5. However it was difficult to identify the compounds in the ethyl acetate scans. It was difficult to differentiate between free pertechnetate and labelled EC-MN with the results obtained. It was however established that the peak of labelled EC-MN does not remain at the origin as with ^{99m}Tc-colloid, when developed in saline and ethanol. A decision was made to perform pertechnetate scans under the conditions of the study rather than base the comparison between pertechnetate and labelled EC-MN on literature only.

3.4.9 Preparing the standard (pertechnetate)

Introduction

The conclusion in the above section (Section 3.4.8) lead to the decision to determine the Rf value of the two pertechnetate sources that were used in the study i.e. that which was eluted at UP/SBAH versus that which was eluted directly from Pelindaba, the manufacturer of ^{99}Mo/^{99m}Tc generators.

Method

Eluted at UP/SBAH

Pertechnetate was eluted from the $^{99}Mo/^{99m}Tc$ generator (NovaTec-P, NTP Radioisotopes (Pty) Ltd, Pelindaba). ^{99}Mo was determined using the dose calibrator. Using a syringe and needle ^{99m}Tc (TcO_4) was spotted onto four ITLC-SG strips and dried before development in acetone, ethyl acetate, and ethanol, respectively. The strips developed in saline were not dried. Each developed strip was scanned in the VCS-201 chromatogram scanner.

Eluted at Pelindaba

A fresh elution of pertechnetate was obtained from NTP Radioisotopes (Pty) Ltd and was delivered to the Nuclear Medicine Dept. at SBAH. Chromatography was performed on this eluate, using four ITLC-SG strips as described above.

This method was repeated the following day with the addition of acetonitrile: water (one to one) as the fifth mobile phase and the results were compared.

Results

Acetone

The scans developed in acetone did not separate well. The peak at the solvent front was broad, with a plateau in Figure 3.11. A peak with a shoulder was observed in Figure 3.12. Both pertechnetate scans showed an undefined Rf value, ranging from 0.6 to 0.7 because of the atypical peaks. These values differ slightly from those documented in Theobald (2011) and the International Pharmacopoeia (W.H.O., 2008) which gives an Rf value of 1.0 for pertechnetate developed in acetone.

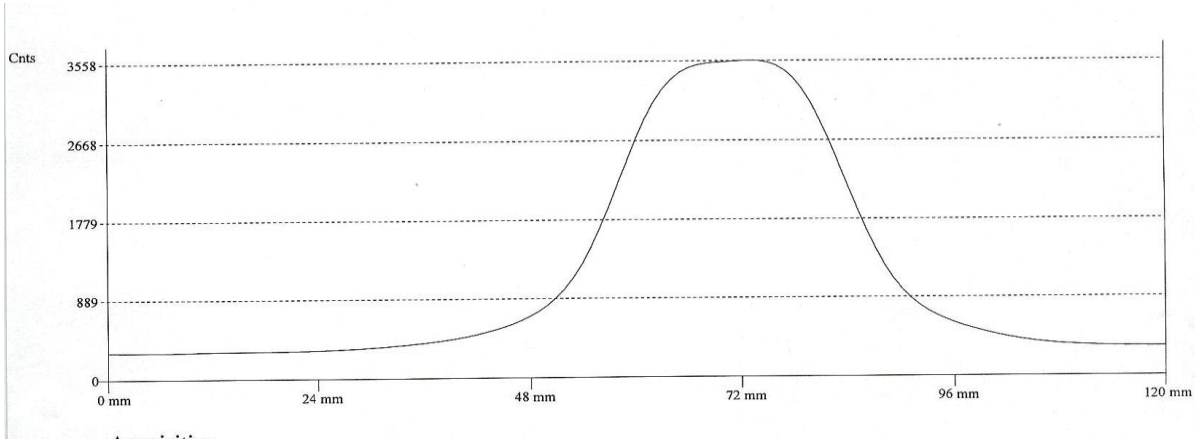


Figure 3.11: Chromatogram of Pelindaba eluted pertechnetate developed in acetone

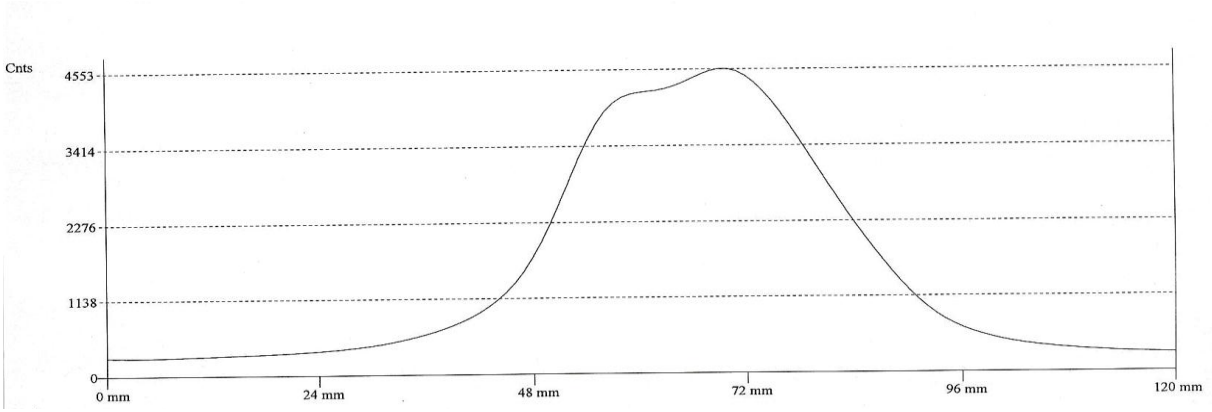


Figure 3.12: Chromatogram of UP eluted pertechnetate developed in acetone

Ethyl acetate

Both chromatograms displayed relatively low residual activity but the peaks were very broad (Figure 3.13 & 3.14). These chromatograms show an Rf value of a compound consistent with that of free pertechnetate. These results, when compared to the ethyl acetate developed ^{99m}Tc-EC-MN chromatograms depicted in Figures 3.2 and 3.8, confirmed that the peak at the origin was not free pertechnetate but, in fact, labelled EC-MN.

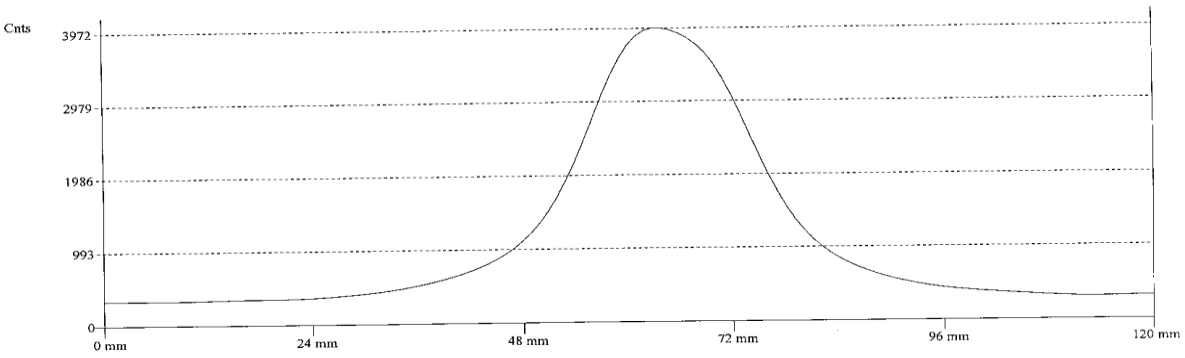


Figure 3.13: Chromatogram of Pelindaba eluted pertechnetate developed in ethyl acetate

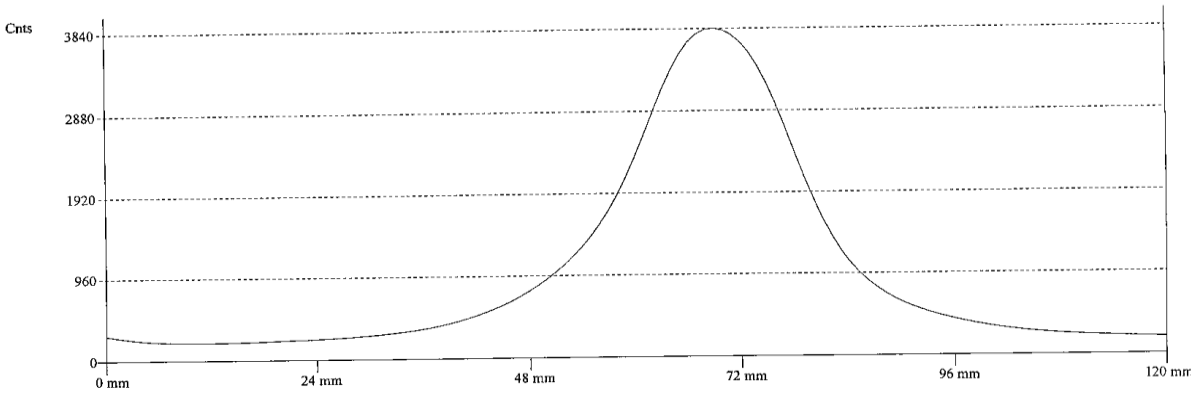


Figure 3.14: Chromatogram of UP eluted pertechnetate developed in ethyl acetate

Ethanol

A broad peak was observed with an Rf value of 0.8 (Figure 3.10).

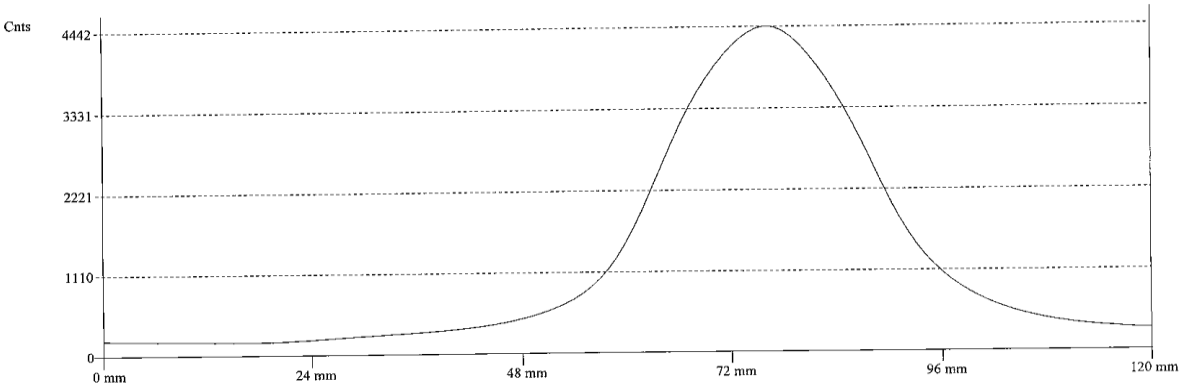


Figure 3.15: Chromatogram of UP eluted pertechnetate developed in ethanol

Saline

A distinct, though broad, peak with low noise was observed at the solvent front, Rf=0.9 (Figure 3.16).

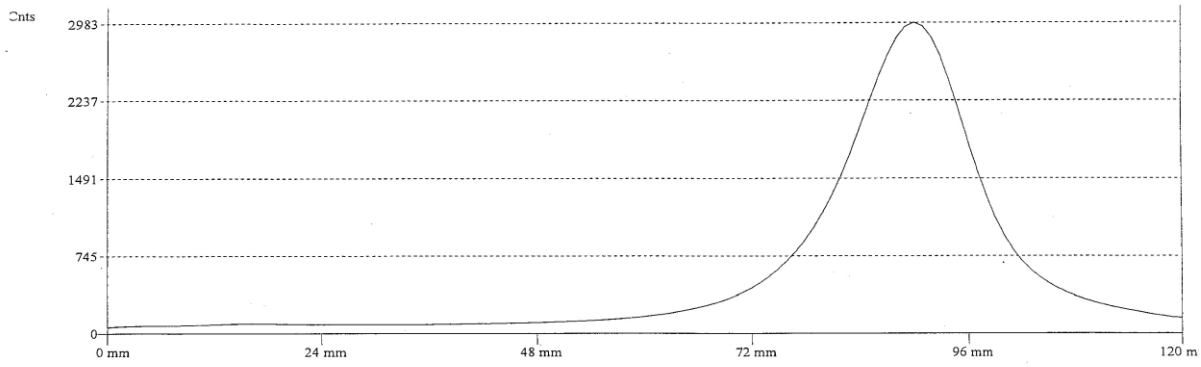


Figure 3.16: Chromatogram of UP eluted pertechnetate developed in saline

Conclusion

The fact that pertechnetate and ^{99m}Tc-EC-MN chromatograms showed similar Rf values posed a challenge in the method of separation suggested by Yang *et al*. (1999). This method does not explain how to differentiate between free pertechnetate and ^{99m}Tc-EC-MN. It is not clear whether there was full labelling, or partial labelling, in which case there would be free pertechnetate which unfortunately migrates with the same Rf.

Separation with ethyl acetate may have been achieved with the ^{99m}Tc-EC-MN peak showing at the origin. The problem was the relatively high residual activity seen with the elution of ^{99m}Tc-EC-MN.

The broad peaks observed may be partly due to the very thin collimator which does not effectively screen the detector (Knoesen, 2011).

3.4.10 Pertechnetate chromatography II

Introduction

Irregular peaks were seen in both the pertechnetate chromatograms that were developed in acetone (Figures 3.11 and 3.12). This phenomenon was a concern because the International

Pharmacopoeia (W.H.O., 2008), which sets the standard for radiopharmacies globally, approves of the use of acetone to develop pertechnetate chromatograms. It was possible that the technique used when spotting pertechnetate onto the chromatography strip may have been inaccurate. To validate the results, the spotting technique was adapted slightly.

Initially a 1 ml syringe and needle had been used to spot pertechnetate onto the ITLC strips. A micropipette was substituted for the syringe and needle to ensure uniformity of size of the droplet, with the aim of producing more accurate and reproducible results.

A comparison of the different pertechnetates from UP and Pelindaba was not performed due to the unavailability of pertechnetate at UP/SBAH at the time of this phase of data collection. Therefore, only Pelindaba pertechnetate chromatography was carried out.

Method

Using pertechnetate eluted from Pelindaba (calibrated to have 60 mCi of activity at 9h00), two sets of chromatography strips (120 mm and 200 mm) were prepared as follows:

Step 1: a) Two µl droplet pertechnetate was spotted on each 120 mm ITLC-SG strip using a micro-pipette.

b) The strips were developed in acetone, saline and ethanol in test-tubes.

c) A few minutes were allowed to ensure that the strips were dry so that they did not wet the scanner plate.

d) The chromatograms were run through the scanner at 120 mm over 60 sec.

Step 2: a) Two µl droplet pertechnetate was spotted on each 200 mm ITLC-SG strip using a micro-pipette.

b) The strips were developed in acetone, saline and ethanol test-tubes.

c) The chromatogram was also run through the scanner at 200 mm in 100 sec.

The droplet size was then increased to 5 µl in an attempt to determine if droplet size affected the peak of an acetone developed chromatogram. The results are shown below.

Results

Acetone

Figure 3.17 shows a broad peak. It is consistent with all the previous pertechnetate scans developed in acetone.

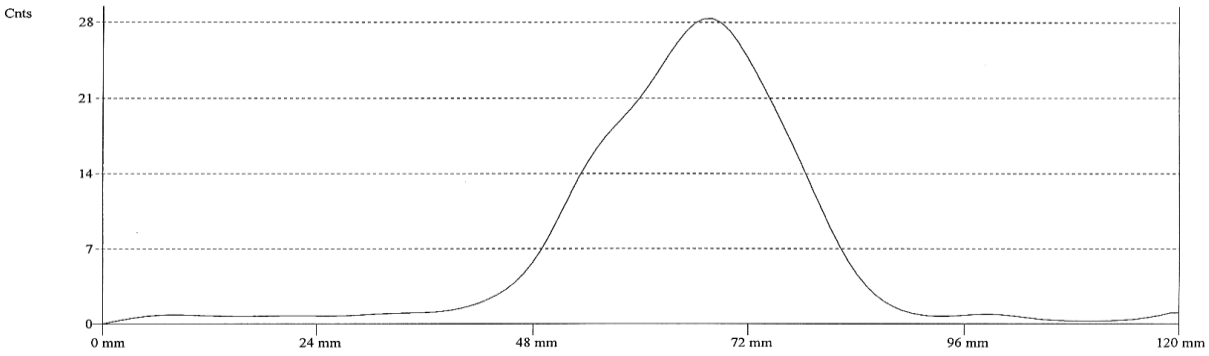


Figure 3.17: Chromatogram of Pelindaba eluted pertechnetate developed in acetone (2 µl droplet size)

Even when scanned at a different rate (200 mm per 100 sec) the acetone-developed scan still proved irregular with a broad peak as seen in Figure 3.18 below.

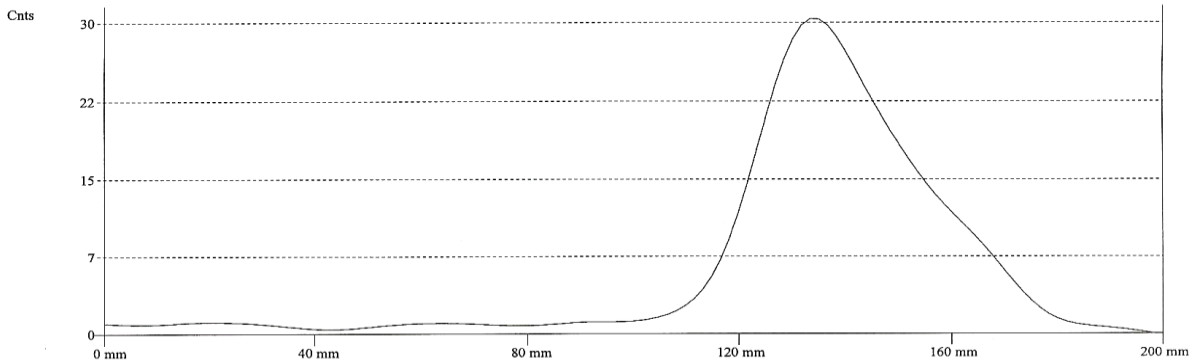


Figure 3.18: Chromatogram of Pelindaba eluted pertechnetate developed in acetone at 200 mm per 100 sec (2 µl droplet size)

Figure 3.19 shows a scan with the larger droplet size of 5 µl. A single peak at the solvent front was observed.

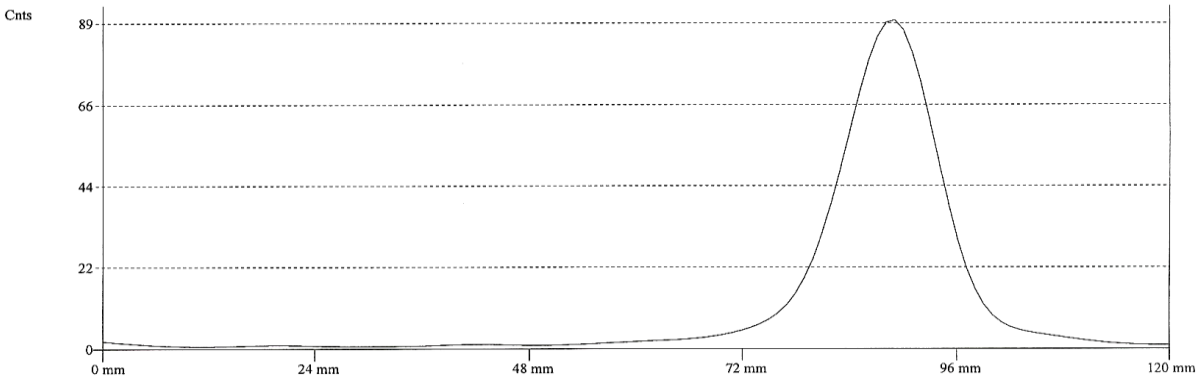


Figure 3.19: Chromatogram of Pelindaba eluted pertechnetate developed in acetone (5 µl droplet size)

Saline

The saline scan in Figure 3.20 below showed no double peak and is similar to the saline scan in Figure 3.16 above.

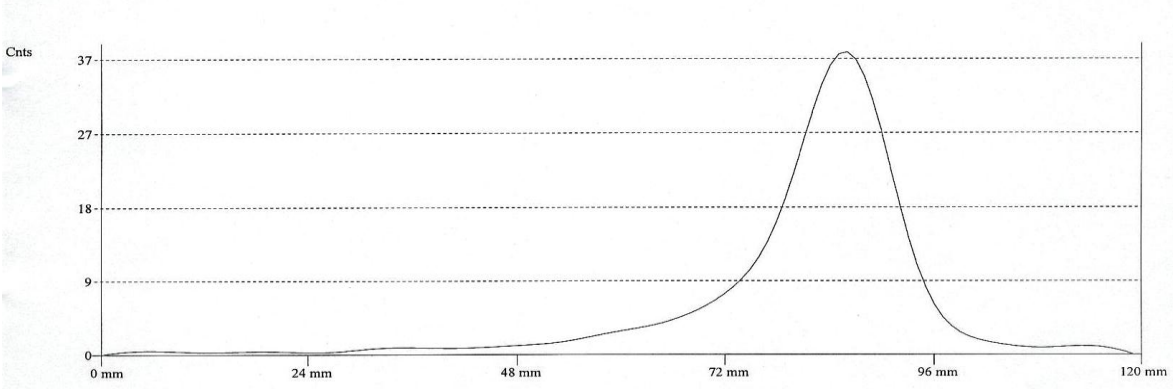


Figure 3.20: Chromatogram of Pelindaba eluted pertechnetate chromatograph developed in saline (2 µl droplet size)

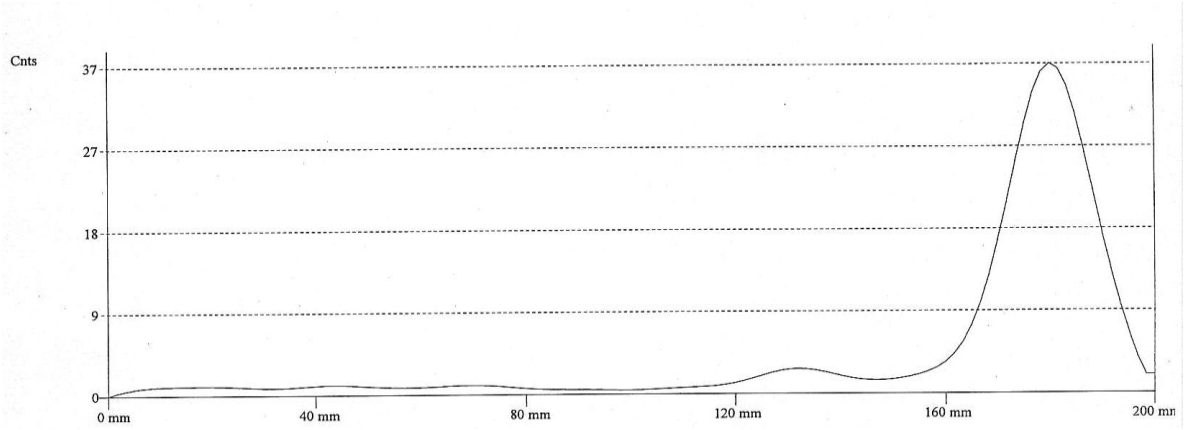


Figure 3.21: Chromatogram of Pelindaba eluted pertechnetate developed in saline at 200 mm at 100 sec (2 µl droplet size)

The diagram above (Figure 3.21) also shows a single peak similar to the one above (Figure 3.20).

Ethanol

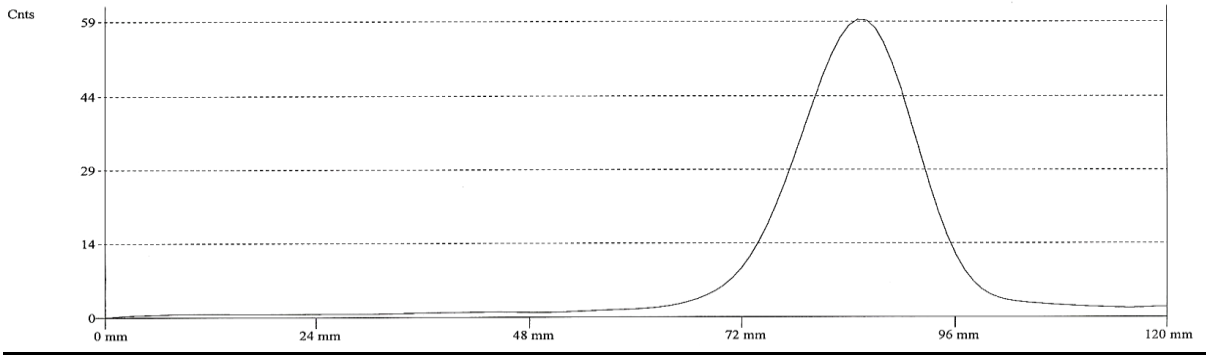


Figure 3.22: Chromatogram of Pelindaba eluted pertechnetate developed in ethanol (2 µl droplet size)

The ethanol scan above (Figure 3.22) shows a single peak similar to the saline scan in Figure 3.10.

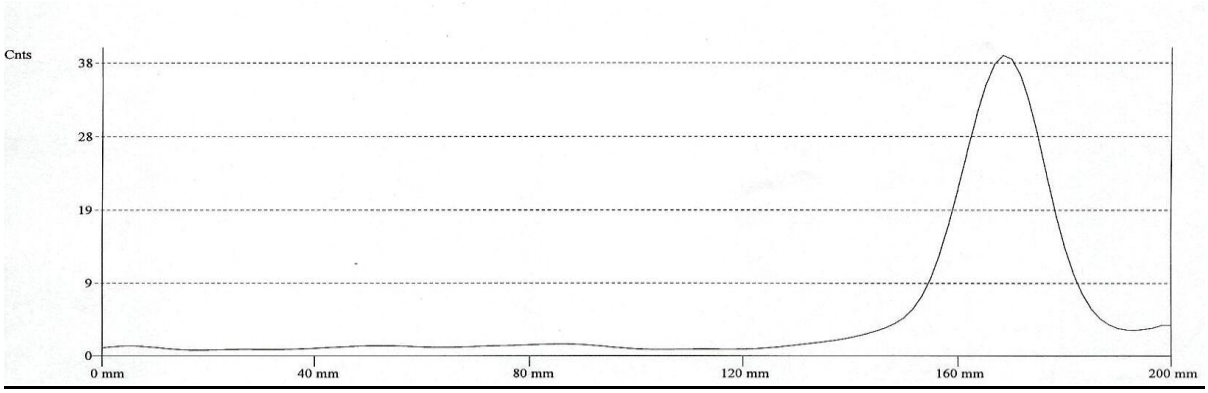


Figure 3.23: Chromatogram of Pelindaba eluted pertechnetate developed in ethanol at 200 mm per 100 sec (2 µl droplet size)

The single peak shown in Figure 3.23 above is similar to the one scanned at 120 mm over 60 sec.

Conclusion

Sample size (droplet) affected the peak of the acetone-developed chromatograms. A large droplet (5 µl) resulted in a chromatogram with distinct separation of components within the compound. Saline and ethanol have proved to be the better two solvents by consistently showing single peaks that were not broad. The above results lead to their selection as solvents for further analysis in the next section (3.4.11).

There was also unacceptable residual activity observed in the ^{99m}Tc-EC-MN chromatograms that were developed in acetone and ethyl acetate. Therefore these two solvents were excluded from further analyses.

3.4.11 Determination of labelling efficiency and stability of expired EC-MN

Introduction

The problem of the broad peaks and the inability to determine whether they were due to poor labelling or the presence of the overlapping product mentioned in the above conclusion, for Section 3.4.10, needed to be solved by using other chromatographic techniques such as reverse phase high performance liquid chromatography (RP-HPLC) coupled to a radioactivity detector and a Diode Array Detector (DAD). These separation techniques were therefore used in an attempt to clarify the efficiency of the EC-MN labelling with

pertechnetate. There was a limited number of sealed vials of EC-MN remaining. Hence it was decided to use the expired Eppindorf tubes of EC-MN for method development to determine the labelling efficiency of EC-MN with pertechnetate as shown by RP-HPLC with a radioactive detector and a DAD. The expired ^{99m}Tc-EC-MN product was also used as a control to determine the stability of the ^{99m}Tc-EC-MN intended for patient use by comparison. The intention of this approach was to establish the Rf value of the newer product and to establish whether it was successfully labelled.

Method

The expired batch of EC-MN vials were analysed using paper chromatography (ITLC-SG), RP-HPLC with a radioactive detector and a DAD in order to compare their stability with that of the newer batch intended for patient administration. The stability results of the expired EC-MN were used as a control.

3.4.11.1 ITLC

Introduction

The chromatograms that were performed in UP/SBAH hot laboratory had broad peaks that may have been due to the type of scanner used or poor labelling. Therefore ITLC was performed again at Pelindaba in order to compare the results. Expired EC-MN was used a comparator to determine if the vial EC-MN produced similar results and thus expired.

Method

Eluted pertechnetate was used to label EC-MN as described in Section 3.4.4. The same method for preparing and spotting ITLC-SG strips described in Section 3.4.5 above, was used to prepare the chromatographs shown below (Figure 3.24- 3.27). Expired ^{99m}Tc-EC-MN was also developed in saline and ethanol.

Results

Saline

A small peak appeared at the origin (Rf=0.0) and the larger peak at the solvent front (Rf=1) (Figure 3.24).

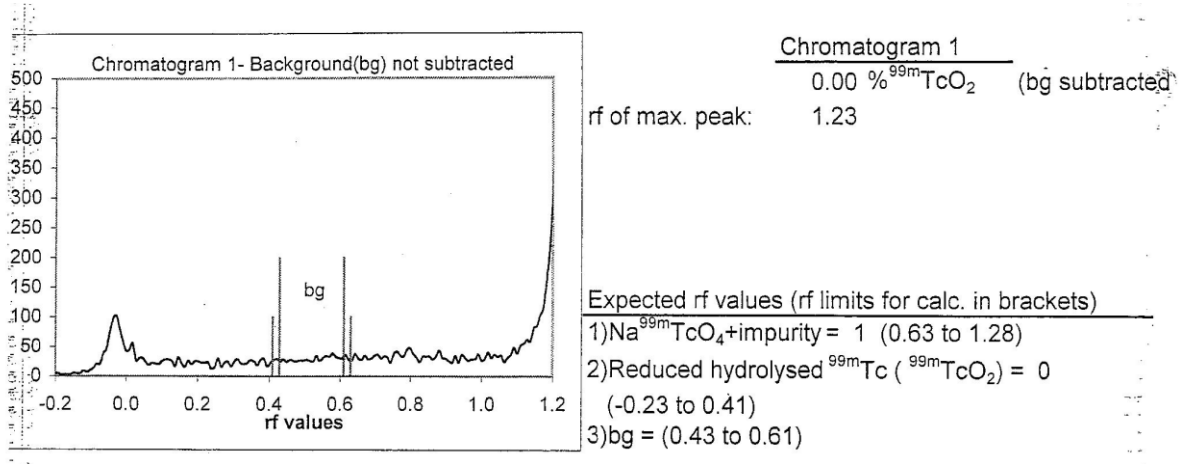


Figure 3.24: Chromatograph of expired ^{99m}Tc-EC-MN (with 0.1 ml $SnCl_2$) developed in saline

Figure 3.25 showed a large peak at the origin (consistent with ^{99m}Tc-colloid chromatograms), when the amount of tin (II) chloride was doubled. The smaller peak at the solvent front revealed that there was a little free pertechnetate or labelled EC-MN. It was difficult to tell which compound was at the solvent front. There was also relatively high residual activity.

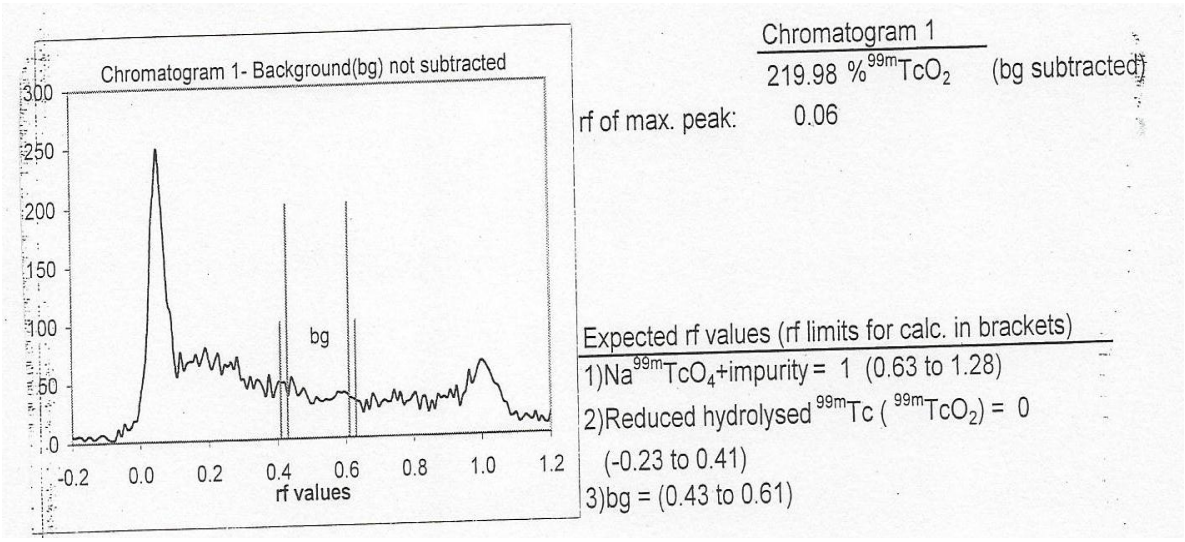


Figure 3.25: Chromatogram of expired ^{99m}Tc-EC-MN (using 0.2 ml $SnCl_2$) developed in saline

Ethanol

When ethanol was used as the solvent, the peak at the origin was larger while the solvent front peak was smaller (see Figure 3.26). This chromatogram shows a lot more ^{99m}Tc-colloid, indicating poor labelling of the expired EC-MN.

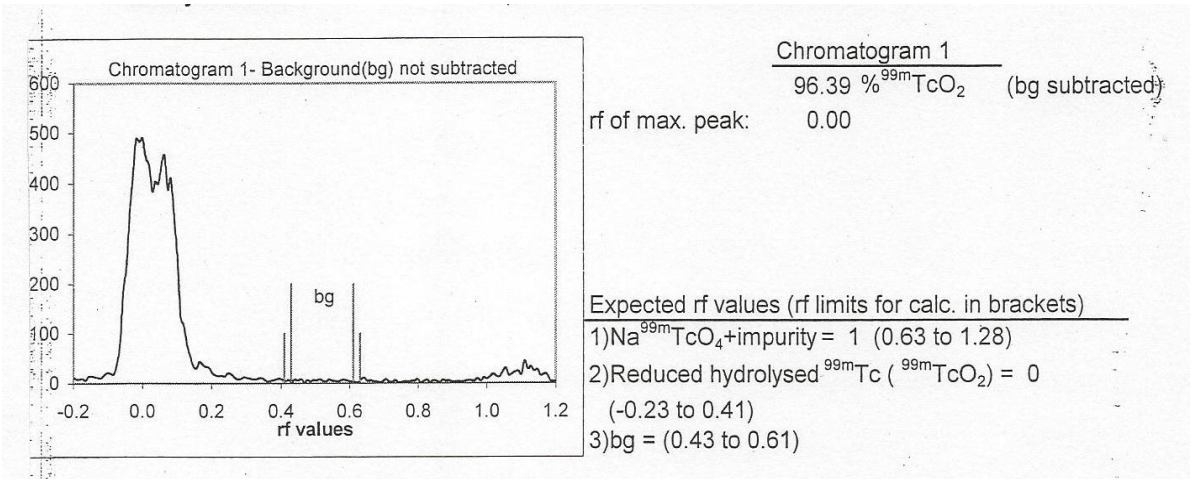


Figure 3.26: Chromatogram of expired ^{99m}Tc-EC-MN (with 0.1 ml $SnCl_2$) developed in ethanol

The ethanol-developed scan (Figure 3.27) also shows a large peak at the origin. This peak indicates that there was ^{99m}Tc-colloid present. There is no peak at the solvent front, hence no free pertechnetate or ^{99m}Tc-EC-MN was present.

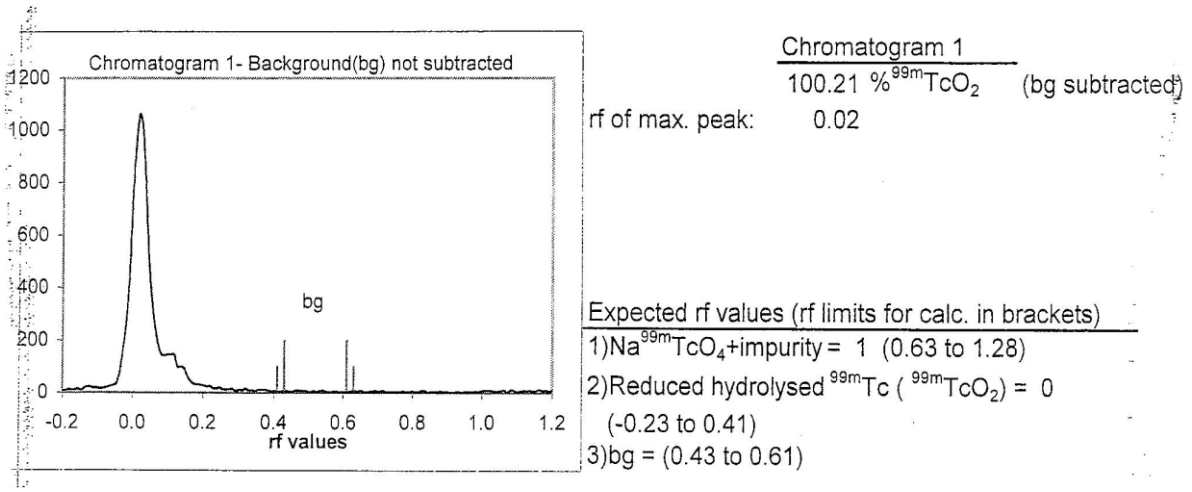


Figure 3.27: Chromatogram of expired ^{99m}Tc-EC-MN (with 0.2 ml $SnCl_2$) developed in ethanol

Conclusion

Both ethanol chromatograms (Figures 3.26 and 3.27) show poor labelling of the EC-MN as the dominant peak was consistent with that of ^{99m}Tc-colloid, seen at the origin. This poor labelling was irrespective of doubling tin (II) chloride in an attempt to improve pertechnetate binding with the ligand. In Figure 3.24 the peak at the solvent front indicates that there may have been some degree of binding or it may have been free pertechnetate, but as tin (II) chloride concentration was increased in Figure 3.25 the poor labelling became evident as demonstrated by the large peak a the origin. It was unclear whether the larger peak at the solvent front represents free pertechnetate or labelled EC-MN. Ultimately, the ^{99m}Tc-EC-MN had poor labelling characteristics, quantitatively. The compound at the solvent front was still undetermined; therefore HPLC was performed (see below).

3.4.11.2 HPLC with Diode Array Detector (DAD)

Introduction

"Reverse-phase chromatography entails chromatographic (RP-HPLC) methods that use a non-polar stationary phase where polar compounds are eluted first while non-polar compounds are retained. Reverse-phase column chromatography accounts for the vast majority of analysis performed in liquid chromatography. The most popular column used is an octadecyl carbon chain (C18) bonded silica. Mixtures of water and aqueous buffers with organic solvents are used to elute analytes from reverse-phase columns.

Elution can be performed isocratically where the water-solvent composition does not change during the separation process or by using a gradient where the water-solvent composition changes during the separation process. The pH of the mobile phase plays a critical role with respect to the retention of the analyte and can change the selectivity of certain analytes" Ntlokwana, 2011.

In the instrumentation used in this analysis, a RP-HPLC was coupled in series fashion with a radioactivity detector (Gabi Star Ray test Gamma Detector) and a DAD. Both the radioactive detector and the DAD use retention time as a measure of chemical separation. A compound that peaks at the same time as another compound, within the same run (under the same conditions), implies that it may be one and the same compound because a different compound would have its peak at a different retention time.

Method

The EC-MN was labelled with pertechnetate as described in Section 3.4.4. The analysis was performed with HPLC coupled with a radioactive detector and a DAD to compare with the results from ITLC. The parameters of HPLC were set as described in the table (Table 3.5) below

RP- HPLC Equipment

"The instrument used was an Agilent 1200 Series Model housing the following modules: A Quaternary pump (G1354A), a Vacuum Degasser (G1322A), a UV-Vis Diode Array Detector (G1315D), a Thermostatted Column Compartment (G1316A) and a Manual Injector (G1328B). Data acquisition was performed by means of single instrument Chemstation software for Liquid Chromatography (LC) system with product number G2170BA. One or more display windows were used to monitor the data acquired by the instrument in real time. An Agilent Zorbax Eclipse XDB-C18 chromatographic column was employed for the separation of the analyte" Ntlokwana, 2011.

The Mobile Phase

"HPLC water was prepared from SG water that was filtered through 0.22µm. This was referred to as the aqueous solvent. An HPLC grade acetonitrile (Merck) was employed as the organic solvent. Several iterative volume combinations of the aqueous solvent and the organic solvent were studied by making use of the quaternary pump. The final mobile phase was optimised to be an isocratic mobile phase made up of a mixture of 85% water and 15% acetonitrile (v/v). The mobile phase mixture contained 1% of tri-ethylamine (TEA) which was added as the ion pairing agent. The pH of the resulting mobile phase was 8.0" Ntlokwana, 2011)

Table 3.5: HPLC analytical method parameters for ^{99m}Tc-EC-MN

Column Name	Agilent XDB Extend C18, 150 mm x 4.6 mm R (5µm)
Column Temperature	30°C
Mobile Phase	Water : Acetonitrile = 85% : 15% (v/v), 1% TEA added as modifier
Mobile Phase pH	8.0
Wavelength	λ = 254 nm
Flow rate	1.0 ml/min
Injection volume	20 µL

TEA = Tri-ethylamine, used as the ion pairing agent.

Ray Test Gamma detector

Data acquisition was performed by means of a GINA Star (version 5.0 Station software).

Due to the urgency to perform the analysis at the time, these chromatographic conditions were not optimised. The peak shapes obtained were not as symmetric, as is ideal; the need at the time was just to establish the radiochemical purity of ^{99m}Tc-EC-MN.

Results

The findings show that the retention time of the 'labelled' EC-MN was approximately 10 min when 0.1 ml and 0.2 ml tin (II) chloride (see Figures 3.28 & 3.29 below) were added to improve labelling.

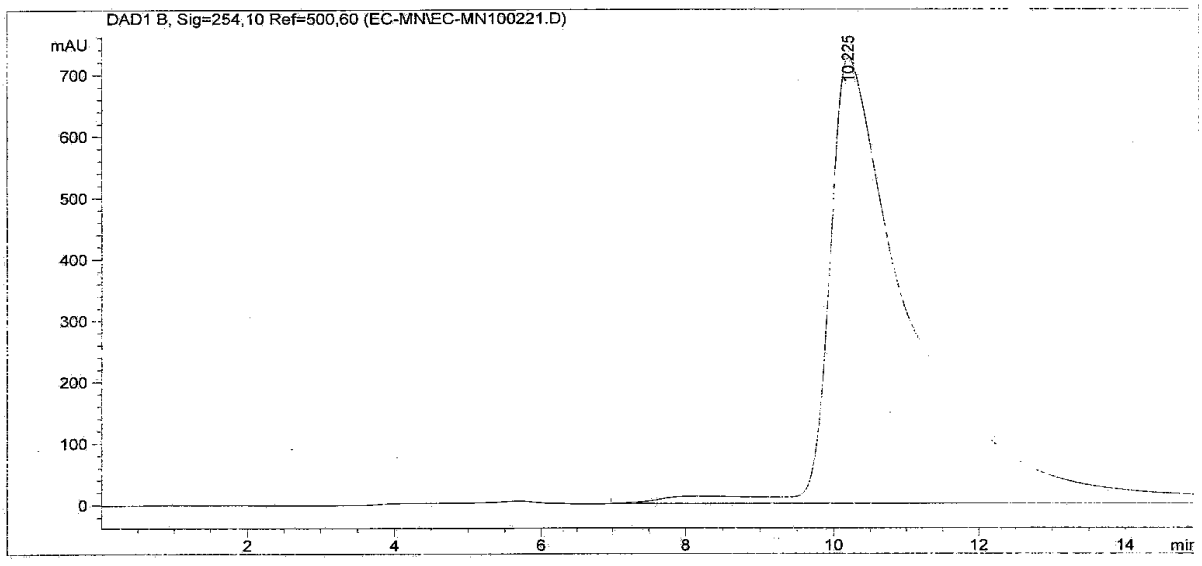


Figure 3.28: HPLC spectrum with DAD of the expired ^{99m}Tc-EC-MN (with 0.1 ml $SnCl_2$)

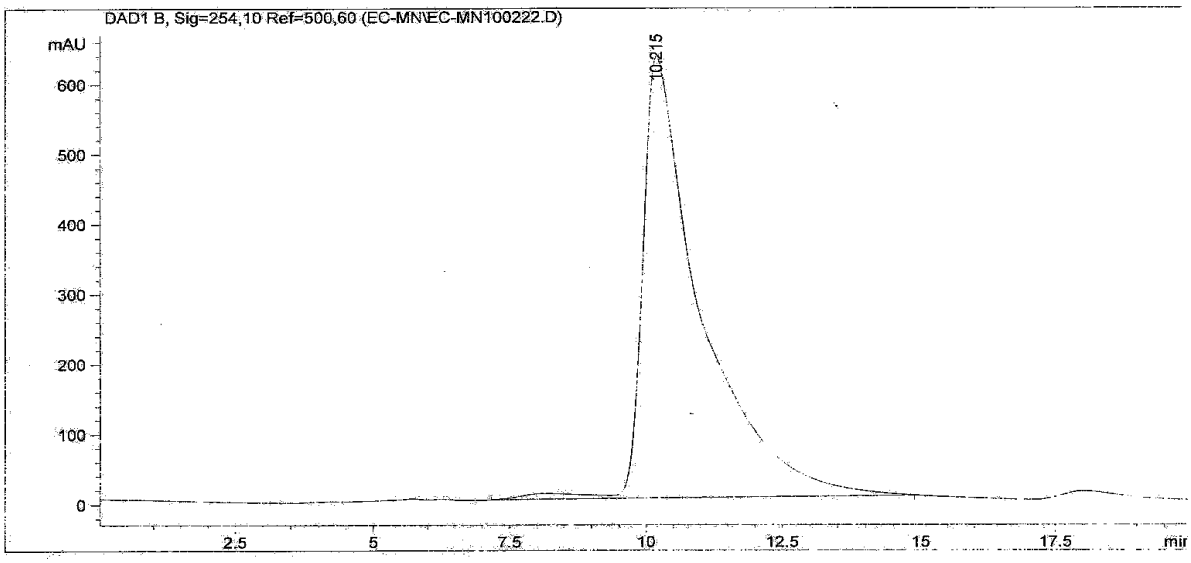


Figure 3.29: HPLC spectrum with DAD of the expired ^{99m}Tc-EC-MN (with 0.2 ml $SnCl_2$)

Conclusion

The similar retention times in the results of the HPLC with DAD scans above depict that there was no difference in the labelling efficiency when double the tin (II) chloride quantity was added.

3.4.11.3 HPLC coupled with a radioactivity detector

Method

Refer to the method explained in sub-section 3.4.11.2 above.

Results

The 'labelled' EC-MN shown in Figures 3.30 & 3.31 below, showed similar retention times (>8 minutes), although the concentration of tin (II) chloride varied (0.1 mland 0.2ml, respectively). These two images also revealed an unbound compound represented by the unshaded areas under the curves just beneath the compound (pertechnetate) represented by the grey shaded area under the curve. The area under curve which represented the unbound compound increased (Figure 3.31) as the concentration of tin (II) chloride doubled.

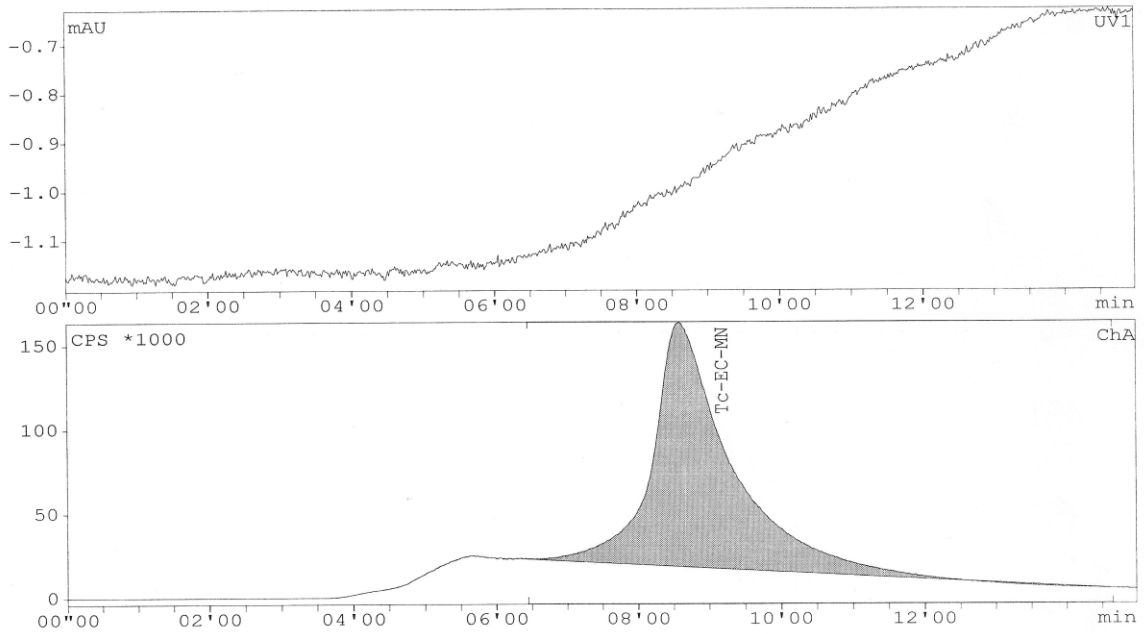


Figure 3.30: Spectrum of the expired ^{99m}Tc-EC-MN (with 0.1 ml $SnCl_2$) using the radioactivity detector

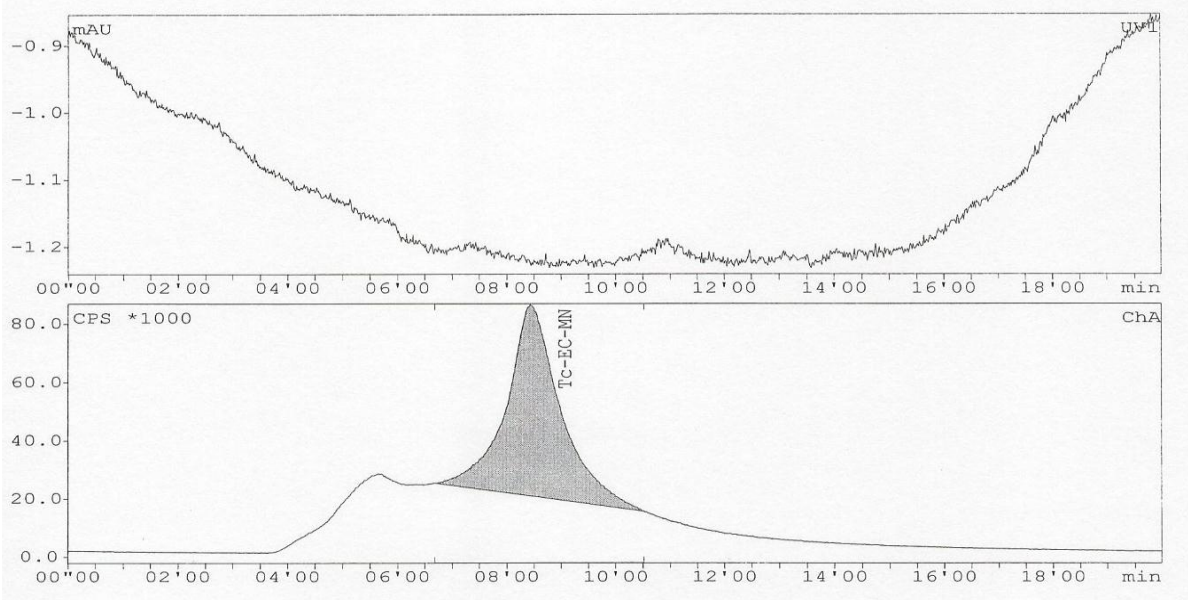


Figure 3.31: Spectrum of the expired ^{99m}Tc-EC-MN (with 0.2 ml $SnCl_2$) using the radioactivity detector

The scan of pertechnetate shown in Figure 3.32 below shows a retention time greater than 8 minutes, as with the expired ^{99m}Tc-EC-MN scan above.

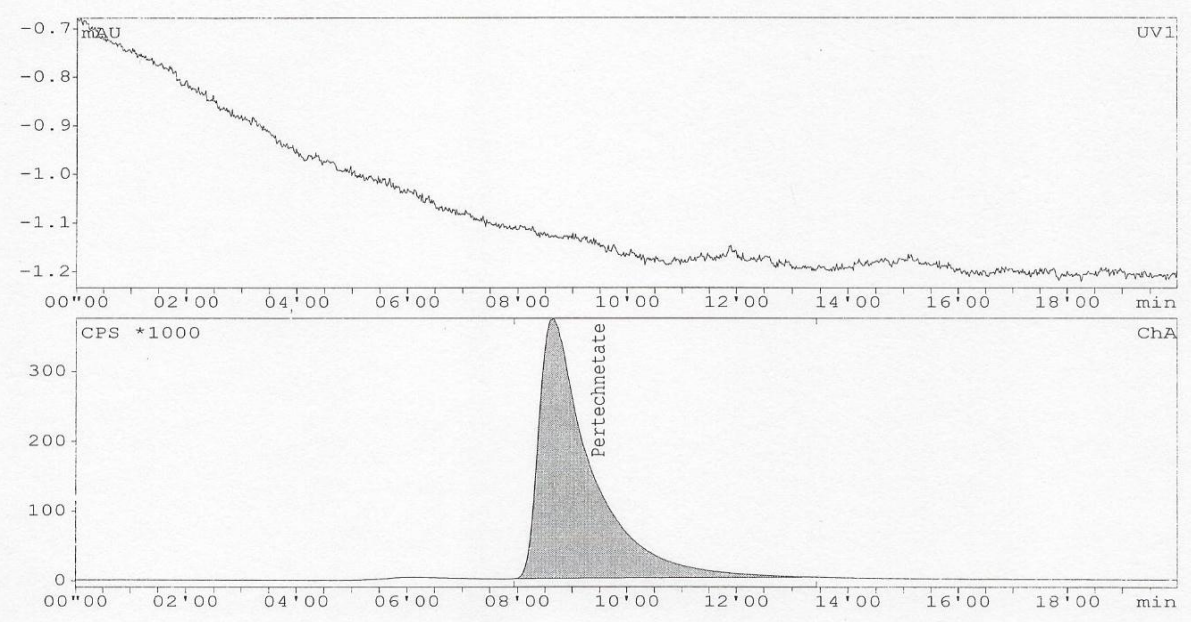


Figure 3.32: Spectrum of pertechnetate using the radioactivity detector

Conclusion

The results indicate that EC-MN was not bound to pertechnetate. Retention times of ^{99m}Tc-EC-MN were the same as pertechnetate, therefore there was no radiolabelling. Increasing the concentration of tin (II) chloride did not improve the labelling either, as the retention times remained the same as that of pertechnetate alone, concurring with the DAD results explained above. It was not possible in the given time to perform further studies so as to establish whether the unbound compound was EC-MN or another product formed from its decomposition products.

3.4.12 Determination of labelling efficiency of vials of EC-MN

Introduction

It was inferred from the results above that the instability of the expired EC-MN had affected its labelling properties. The aim therefore was to establish whether the newer vials of EC-MN had degraded or not, based on their ability to bind to pertechnetate.

3.4.12.1 ITLC

Method

See method under ITLC in Section 3.4.10.

Results

EC-MN was labelled with pertechnetate using double the amount of tin (II) chloride and the chromatograph was developed in saline (top) and ethanol (bottom) (Figure 3.33). A single peak appeared at the solvent front (Rf=1.0) in the saline profile while the two peaks (origin and solvent front) were observed in the ethanol profile.

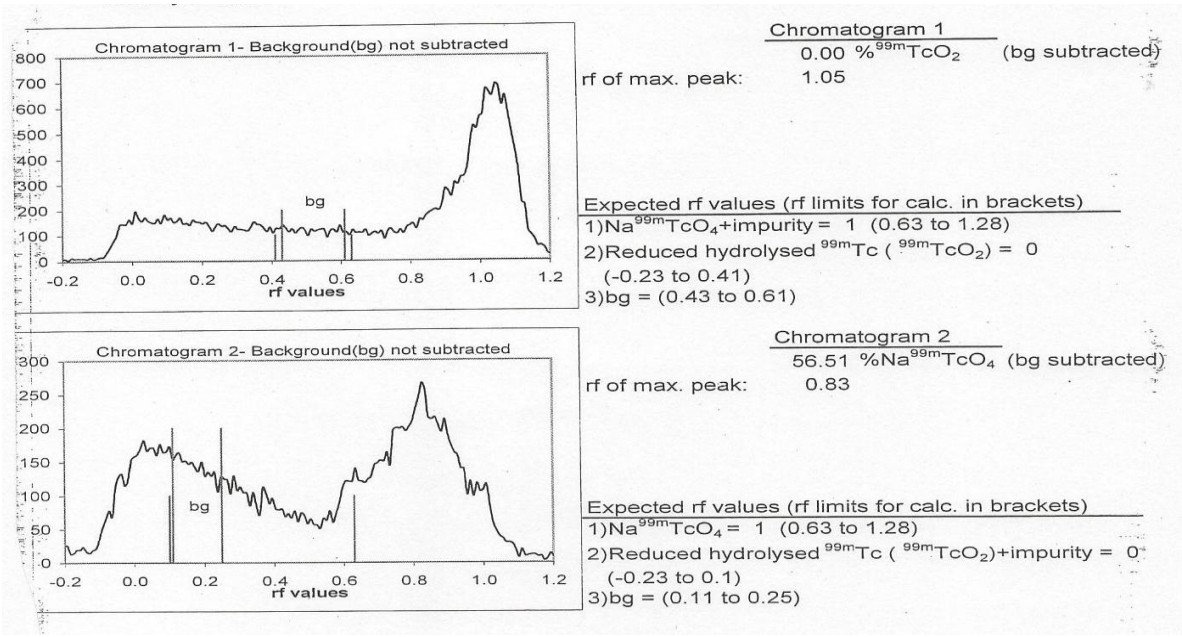


Figure 3.33: Chromatograms of vial ^{99m}Tc-EC-MN (with 0.2 ml $SnCl_2$) developed in saline (top) and ethanol (bottom)

Conclusion

It was still unclear whether the single peak observed in the top chromatogram was pertechnetate alone or ^{99m}Tc-EC-MN. The lower image in Figure 3.33 however shows a negative effect from the doubled tin (II) chloride quantity, with multiple peaks. However the difference in the Rf values of the solvent front peaks in both chromatograms (top and bottom) indicate that these may represent different compounds. The peak at the origin (Rf=0.0) in the ethanol-developed chromatogram (bottom) is consistent with ^{99m}Tc-colloid in Figure 3.25 above, and the solvent front peak, ^{99m}Tc-EC-MN or free pertechnetate. However,

a comparison of all the ethanol-developed chromatograms with those that were developed in saline suggests that ^{99m}Tc-EC-MN migrates to the solvent front with both solvents. Figure 3.33 was the exception which means that the peak observed at the origin may have been residual activity and not ^{99m}Tc-colloid. Neither of these images showed separation nor a peak that indicated a new product that might have been ^{99m}Tc-EC-MN labelling.

3.4.12.2 HPLC with Diode Array Detector

Method

Refer to method in sub-section 3.4.11.2 above.

Results

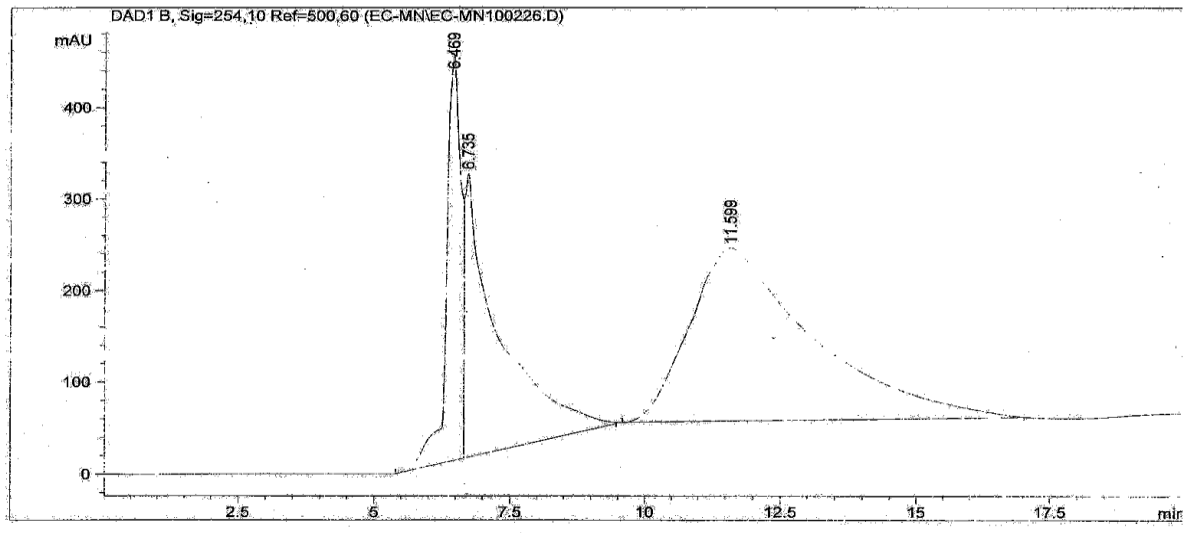


Figure 3.34: Spectrum of vial ^{99m}Tc-EC-MN analysed with HPLC coupled with diode array detector

Analysis with DAD showed a labelled compound at approximately 6.5 minutes (Figure 3.34), This retention time differed from the peaks viewed in Figures 3.28 and 3.29 (retention times of 10.225 and 10.215 minutes respectively). A large peak with a retention time of 11.599 was also observed.

Conclusion

The compound that peaked at 6.5 minutes in the above image (Figure 3.34) is not pertechnetate. It is unclear what the larger peak represents.

3.4.12.3 HPLC coupled with a radioactivity detector

Method

Refer to method in sub-section 3.4.11.2 above.

Results

Analysis with the radioactivity detector showed the peak at a retention time that is less than 8 minutes (Figure 3.35).

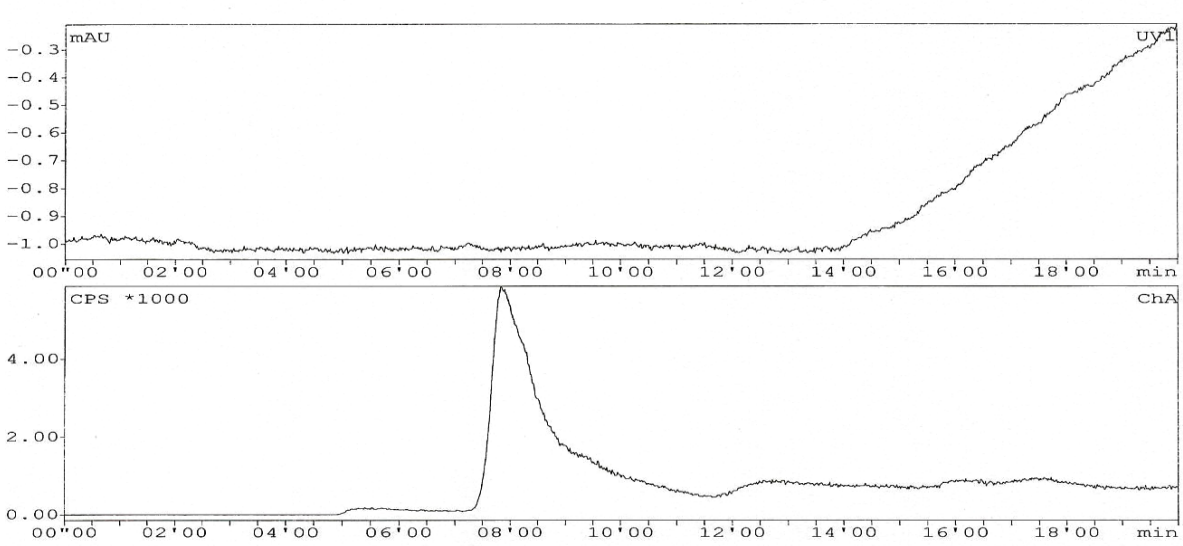


Figure 3.35: Spectrum of vial ^{99m}Tc-EC-MN using HPLC coupled with a radioactivity detector

Conclusion

A comparison of the retention time of the peak in Figure 3.35 with retention time of the pertechnetate peak in Figure 3.32 indicates that the peak in Figure 3.35 was due to a compound other than pertechnetate, i.e. the ^{99m}Tc-EC-MN. The above findings indicated that the vial of EC-MN had been successfully labelled which indicated that it was stable. Based on these results and those in sub-section 3.4.12.2 it was agreed that the remaining vials EC-MN could be used to prepare dose for administration to patients.

3.4.13 Chromatography of ^{99m}Tc-EC-MN (Patient 1)

Introduction

The work described in sub-section 3.4.12.2 provided evidence that the EC-MN had been successfully labelled with pertechnetate. Hence preparation of the patient dose went ahead in order to obtain SPECT images for hypoxic cervical cancer tissue.

As specified in the cGRPP (Elsing *et al*., 2010) radiopharmaceuticals intended for patient administration must undergo quality control procedures before they are issued to be injected into the patient.

Method

Eluted pertechnetate was used to label EC-MN as described in Section 3.4.4. The same method for preparing and spotting on ITLC-SG strips as described in Section 3.4.5 above, was used. IITLC was performed on ^{99m}Tc-EC-MN with the use of ethanol and saline as the solvents. It was filtered with 0.22um filter thereafter to sterilise the preparation.

Results

Although a peak was observed at the solvent front, the chromatogram below (Figure 3.36) showed a great deal of residual activity, towards the origin.

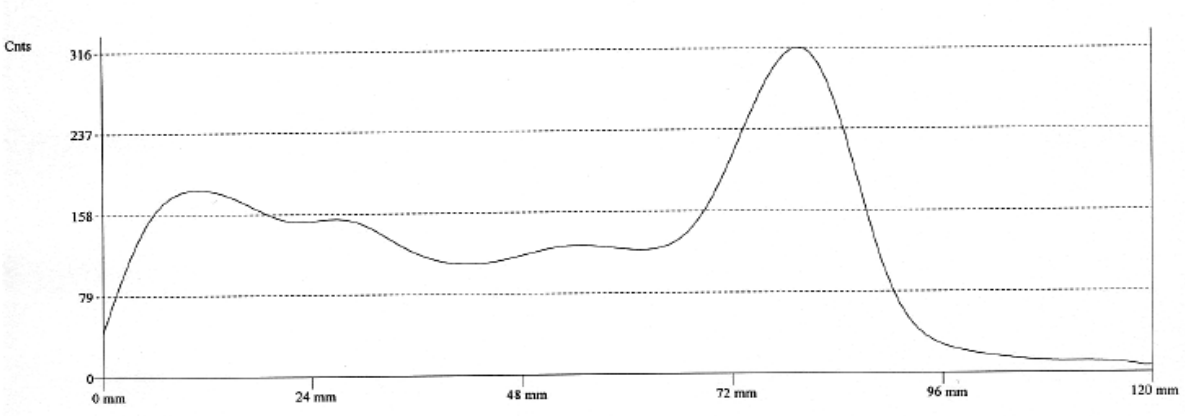


Figure 3.36: Chromatogram of ^{99m}Tc-EC-MN developed in ethanol for Patient 1

The saline-developed scan (Figure 3.37) also showed some activity at the origin, indicative of the presence of residual activity on the chromatogram. The labelled product was at the solvent front (Rf=0.7).

The degree of labelling could not be established but it was below 95% and differed slightly from the product developer's scan from pilot data which had labelling efficiency greater than 95% (Figure 3.38). There were two main differences; i) the use of a Veenstra chromatogram scanner (VCS 201, Veenstra, Netherlands) in this study as opposed to the radio-TLC scanner (Bioscan, Washington DC) used by the product developer and ii) the use of ITLC-SG paper when the developer did not specify which type of plates were used.

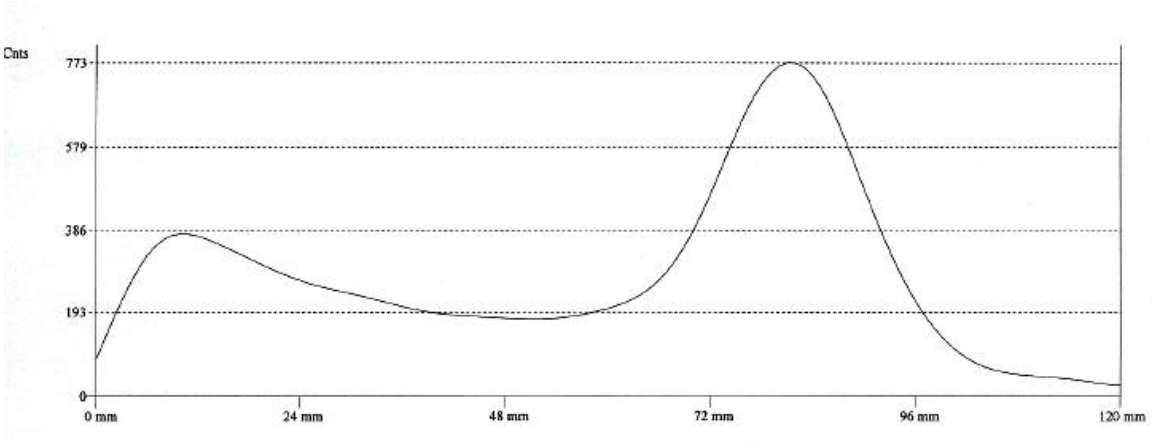
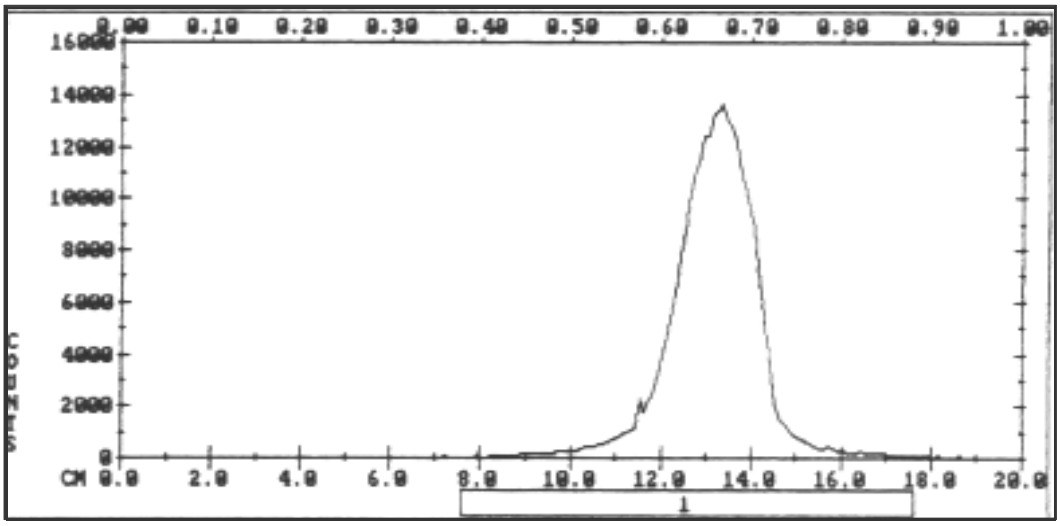
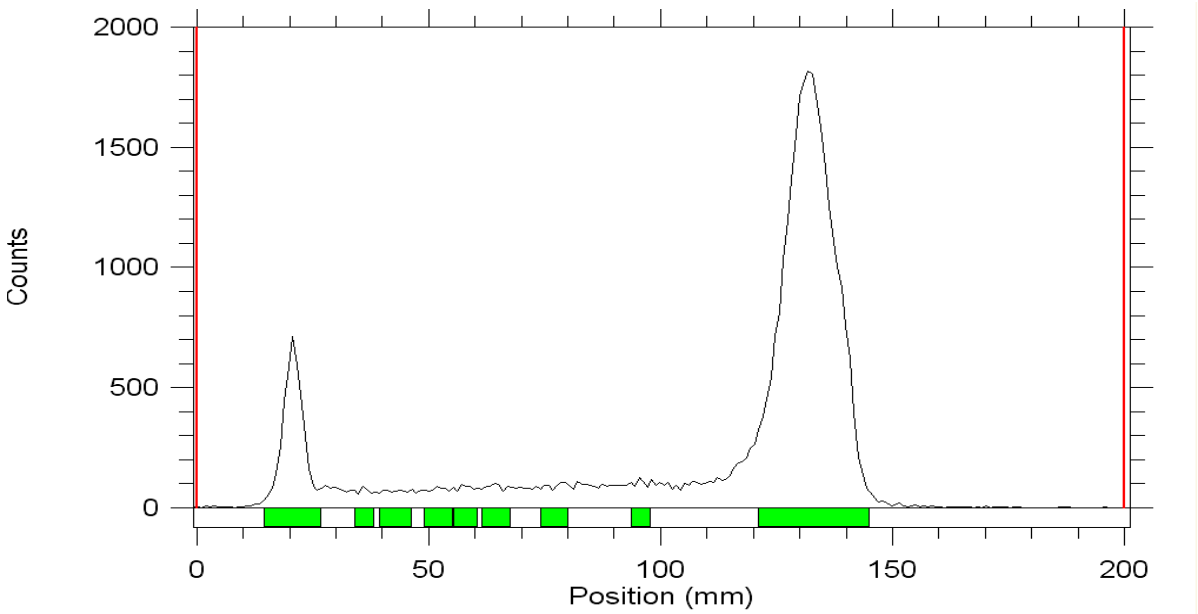


Figure 3.37: Chromatogram of ^{99m}Tc-EC-MN developed in saline for Patient 1



Source: Pilot data from D Yang, PhD. The University of Texas, M.D. Anderson Cancer Centre Houston, Texas. Received 2009.

Figure 3.38: Radio-TLC chromatogram of ^{99m}Tc-EC-MN (saline) 1



Source: Pilot data from D Yang, PhD. The University of Texas, M.D. Anderson Cancer Centre Houston, Texas. Received 2009.

Figure 3.39: Radio-TLC chromatogram of ^{99m}Tc-EC-MN (saline) 2

Successfully labelling EC-MN was a common challenge, even for the product developer. Figure 3.38 shows a good product yield as there is no activity seen at the origin whereas the radiochemical purity of ^{99m}Tc-EC-MN shown in Figure 3.39 indicates that there were variable results, some of which were below 95%.

Conclusion

EC-MN was assumed to be successfully labelled and was administered to the patient

3.4.14 Chromatography of ^{99m}Tc-EC-MN (Patient 2)

Introduction

The last vial of EC-MN was used to prepare the dose of ^{99m}Tc-EC-MN for Patient 2. It had been four months since the chromatography of Patient 1, so further degradation of EC-MN may have occurred. This patient's tumour was suspected to be hypoxic.

Method

Eluted pertechnetate was used to label EC-MN as described in Section 3.4.4. The same method for preparing and spotting on ITLC-SG strips as described in Section 3.4.5 above,

was used. ITLC was performed on ^{99m}Tc-EC-MN with the use of ethanol and saline as the solvents.

Results

The chromatograms of both ethanol and saline (Figures 3.40 and 3.41) showed residual activity that was very high, which is also unacceptable. There was a large, very broad peak at the origin. The ligand, EC-MN, was unable to form a bond with reduced pertechnetate to yield ^{99m}Tc-EC-MN resulting in a large quantity of ^{99m}Tc-colloid (Rf=0.0).

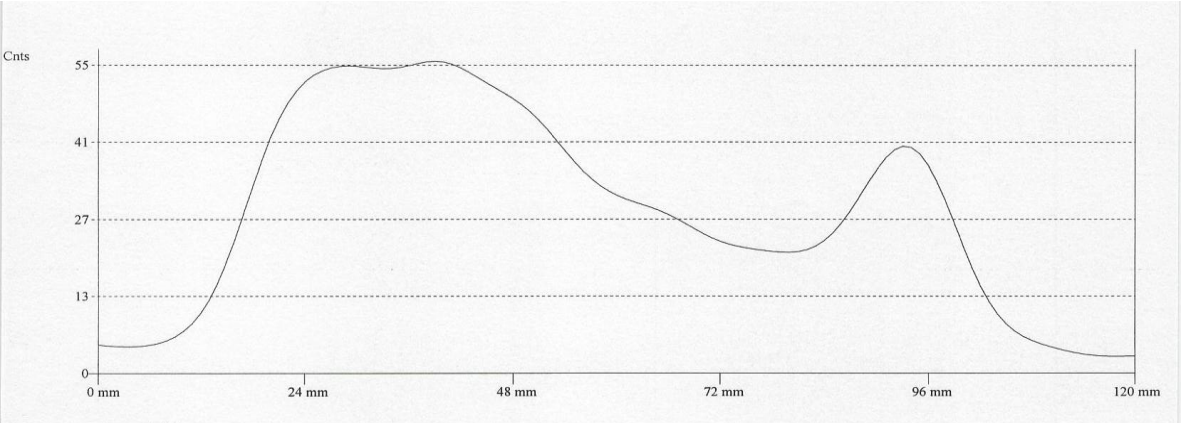


Figure 3.40: Chromatogram of ethanol developed ^{99m}Tc-EC-MN prepared for Patient 2

In addition, when ^{99m}Tc-EC-MN was filtered with 0.22 µm filter to sterilise the preparation, the radiopharmaceutical would not pass through the filter. The back pressure resulted in a spill of the preparation. When a second filter was attached, the same event occurred. There was then too little ^{99m}Tc-EC-MN left for use. The chromatograms were run through the scanner 24 hours later as there was sufficient activity remaining on the chromatogram. Furthermore, it also took a little longer than normal (more than 10 min) to dissolve the EC-MN in the vial before filtering it, which served as warning that the product had further degraded.

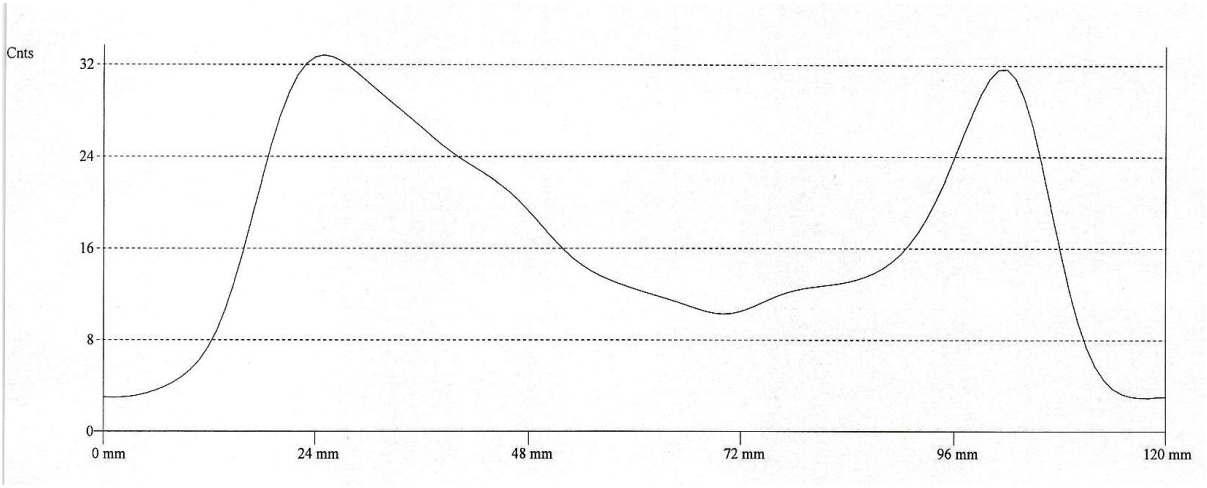


Figure 3.41: Chromatogram of saline developed ^{99m}Tc-EC-MN prepared for Patient 2

Conclusion

A comparison of this ^{99m}Tc-EC-MN with the previous one of Patient 1 (Section 3.4.13) suggests that the particular vial of the ligand (EC-MN) had decomposed to form other compounds that would not pass through the filter. The chromatograms also show very poor labelling yields with $^{99m}TcO_2$ (^{99m}Tc-colloid) as the predomination ion in the solution. There may also have been a stability difference between the vials as the product was not manufactured under GMP principles. The study on the patient was thus aborted.

CHAPTER 4
CLINICAL APPLICATION OF ^{99m}Tc-EC-MN: METHOD, RESULTS & DISCUSSION

4.1 INTRODUCTION

Due to the logistical constraints explained in the Introduction of Chapter 3, only two of the original 6 vials of product were left for administration to patients. Logistical delays lead to a time lag of four months before the second patient was imaged. As a result further degradation of the EC-MN may have taken place. This chapter provides a case report on the only patient who was imaged.

4.2 ETHICAL CONSIDERATIONS

The study was approved by the ethics committee of the University of Pretoria (UPREC) and by the Medunsa Research and Ethics Committee (MREC) of the University of Limpopo (Medunsa campus). The certificates of approval are attached in Appendix 1 and Appendix 2, respectively. The data were collected using a Case Record Form (see Appendix 3) and written consent was obtained from each patient after informing them regarding the study, in the language of their choice as far as possible. See attached consent form in Appendix 4.

4.3 CASE STUDY

Two newly-diagnosed patients with cervical cancer were prospectively recruited. They were selected according the criteria tabulated below (Table 4.1). Each patient was to receive a ^{99m}Tc-EC-MN scan before radical hysterectomy and lymph node dissection (RHLND). Only one patient received ^{99m}Tc-EC-MN scan results which were evaluated and compared to the classical work-up (demonstrated below) and the clinical follow-up.

Table 4.1: Inclusion and exclusion criteria

Inclusion criteria
• First diagnosis of histologically proven squamous cell cervical cancer
• Locally advanced operable cervical cancer FIGO stages IB2 and IIA.
• Consent to participate
• Age < 70 years
• Suitable for MRI and SPECT
Exclusion criteria
• Pregnant patient
• Breast-feeding women,
• Patients who are not able to lie motionless for one hour.
• Patients on neoadjuvant chemotherapy,
• Other pre-existing malignancies, age > 70 years,

4.3.1 Clinical data for Patient 1

4.3.1.1 Patient History

A 49 year old female patient was referred from Rob Ferreira Hospital with a diagnosis of a stage IIa1 cervical cancer. Her main complaint was vaginal bleeding for 14 months. She was booked for radical hysterectomy which includes the removal of the pelvic lymph nodes, the cervix and the uterus, the day after imaging.

4.3.1.2 Diagnosis of cervical cancer

Table 4.2: Biopsy of cervical cancer

Date of cervical biopsy	28 October 2010
Diagnosis	Invasive keratinising squamous cell carcinoma grade II High grade SIL and condylomatous change
Tumour size	1 x 0.5 x 0.5cm
Lymphovascular involvement	None
Grade	II

4.3.1.3 Blood investigations

Table 4.3: Pre-operative full blood count

HIV status	Negative	
CD 4 count	987 x 10^6/l	
Full blood count	Red cell count	4.60 x 10^{12}/l
	Haemoglobin	13.6 g/dl
	Haematocrit	0.430 l/l
	MCV	93.7 fl
	MCH	29.5 pg
	MCHC	31.5 g/dl
	Red cell distribution width	16.7 %
	Platelets	258 x 10^9/l
	White cell count	6.89 x 10^9/l
Kidney function test	Sodium	139 mmol/l
	Potassium	4.5 mmol/l
	Chloride	106 mmol/l
	Carbon dioxide	16 mmol/l
	Urea	4.0 mmol/l
	Creatinine	62 mmol/l
	MDRD	>60 ml/min/1.73m^2
	Anion Gap	22 mmol/l
Liver function test	Bilirubin total	7 µmol/l
	Albumin	40 g/l
	Alkaline phosphatase	65 U/l
	γ–glutamyl transferase	37 U/l
	Alanine transaminase	15 U/l
	Aspartate transaminase	21 U/l

The full blood count displayed in Table 4.3 above showed normal levels of haemoglobin which carries oxygen in blood. The normal haemoglobin indicated that the oxygen carrying capacity of blood was not compromised.

4.3.1.4 Routine imaging procedures

Table 4.4: Ultrasonography and X-ray results

Ultrasonography	
Kidneys	Normal; • No masses • No hydronephrosis • No renal calculi
Bladder	Normal; • No obvious masses seen • Bladder wall not elevated
X-ray	
Chest x-ray	Normal; • Central trachea • Normal cardiac shadow size • No hilar lymph adenopathy • Bilateral lung fields are clear • No pleural effusion • Bony element appear normal • Soft tissue shadows appear normal

Results from ultrasonography showed that there was no evidence to suggest obstructive uropathy. In Table 4.4 the x-ray findings are shown, which proved normal with no evidence of pulmonary metastases. No masses or abnormal tissue appeared.

The patient was referred from another hospital where MRI and CT scans were expected to have been performed in order to diagnose the patient. These studies were therefore omitted at SBAH as the diagnosis was already made. This unfortunately left a gap in the investigation of this study, as those scans were not available for interpretation for the evaluation of hypoxia..

The table below (Table 4.5) details the histological evaluation post radical hysterectomy and lymph node dissection. Macroscopic and microscopic evaluation revealed that the cervical tumour had invaded 90% of the cervical wall thickness and it extended very close to the resection margins. Three of the eight identified pelvic lymph nodes had metastatic tissue and the endometrium had a proliferative appearance. The parametria, Pouch of Douglas and adnexia were all tumour free. No lymphovascular involvement was noted.

Because there was only one patient left to investigate, it was not an economic option to purchase the VEGF and MVD kits which are estimated between R10 000 – R20 000. The tests were therefore not performed. These tests would have provided information on the hypoxic state of the tumour and would have been the standard of comparison for the scintigraphic scans. As a result, the absence or presence of hypoxia could not be reliably established. Therefore, there was the possibility of a false negative scan from the scintigraphy.

4.3.1.5 Histological evaluation

Table 4.5: Histological and immunohistochemical results

<table>
<tr><td rowspan="2">Routine staging and tumour characteristics</td><td colspan="3">Cervical carcinoma stage IIa</td></tr>
<tr><td colspan="2"><u>Macroscopy</u>
Cervical tumour involves proximal endocervical canal and lower uterine segment.
Tumour size = 4x2x2cm
Large left pelvic lymph nodes (6); 2 nodes contain metastatic carcinoma.
Right pelvic lymph nodes (2); 1 contains metastatic carcinoma.</td><td><u>Microscopy</u>
Section of uterus show poorly differentiated squamous cell carcinoma of the cervix.
Tumour involves approx. 90% of wall thickness of cervix and extends to within 1mm from the resection margin.
In situ carcinoma extends very close to vaginal cuff resection margin.
Invasive carcinoma involves endocervical canal and lower uterine segment.
Maximum thickness of tumour approx. 12mm.
No lymphovascular invasion noted.</td></tr>
<tr><td>Immunohistochemical measures of hypoxia [vascular endothelial growth factor (VEGF)]</td><td colspan="3">Not performed due to financial constraints</td></tr>
<tr><td>Histological measures of hypoxia using tumour vascularity [microvessel density (MVD)]</td><td colspan="3">Not performed due to financial constarints</td></tr>
<tr><td rowspan="5">Diagnosis</td><td>Cervix</td><td colspan="2">Poorly differentiated squamous cell carcinoma which involves the lower uterine segment

While the resection margins appear tumour free, tumour is noted very close to the margins</td></tr>
<tr><td>Parametria</td><td colspan="2">Tumour free</td></tr>
<tr><td>Pouch of Douglas</td><td colspan="2">Tumour free</td></tr>
<tr><td>Adnexia</td><td colspan="2">Tumour free</td></tr>
<tr><td>Pelvic lymph nodes</td><td colspan="2">Metastatic carcinoma (3/8)</td></tr>
</table>

4.3.1.6 Post-surgical intervention

The patient was discharged from hospital and then referred for further intervention with chemotherapy and radiation. She was admitted a month later to start on the treatment. The prescribed irradiation treatment was 46 Gy total doses in 23 cycles, five times a week. She also underwent 11 Gy total doses in 2 cycles brachytherapy once a week and one course of chemotherapy with cisplatin 30 mg/m^2. The patient was discharged after apparent successful completion of her treatment.

4.3.1.7 Conclusion

The blood investigation before surgery showed that the patient had haemoglobin levels that were within range suggesting normal oxygenation. The histological report stated that there was no lymphovascular involvement.

4.3.2 Tumour hypoxia imaging using ^{99m}Tc-EC-MN with SPECT

4.3.2.1 Indication

Patient 1 was administered ^{99m}Tc-EC-MN for detection of possible hypoxia, prior to surgery.

4.3.2.2 Radiopharmaceutical and Procedures

Table 4.2 shows the protocol followed when imaging the patient. 30 mCi ^{99m}Tc-EC-MN was injected IV followed by dynamic images (blood-flow and -pool), whole body static images (planar), delayed SPECT images. The images were obtained with the use of a dual headed camera (Infinia). A low energy, high resolution collimator was used for each study.

Table 4.6: Protocol followed for imaging tumour hypoxia in cervical cancer

Type of study	Type of view	Duration	Acquisition time
Anterior pelvis flow	Anterior pelvis	60 sec: 30 frames @ 2 sec/frame	Immediately post injection
Blood pool pelvis static	Anterior & posterior	750 Kcounts	3 min post-injection
Pelvis static	Anterior & posterior	750 Kcounts	30 min post-injection
Thyroid static	Anterior	750 Kcounts	30 min post-injection
SPECT	Pelvis	25 sec/view: 60 views with 6º interval	40 min post-injection
Whole body scan	Anterior & posterior	12 cm/min	60 min post-injection

The patient was asked to empty her bladder before the delayed images were obtained in order to view accumulation of the tracer in the pelvis. No other specific patient preparation was performed except for briefing about the procedure when consent was obtained.

4.3.2.3 Results

Blood flow

The blood flow images were acquired while the tracer was injected in the patient's arm. As depicted in Figure 4.1 below, the tracer was visible in the renal blood circulation by the fourth frame (see arrow).

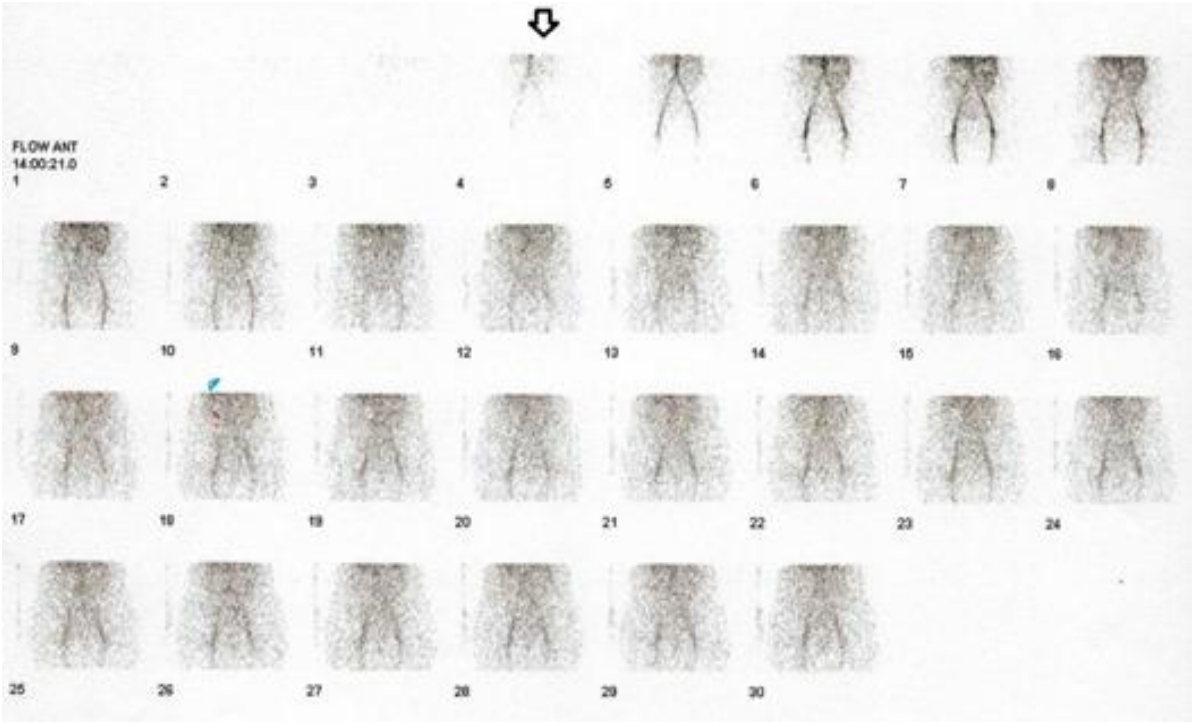


Figure 4.1: Anterior pelvis blood flow image acquired while injecting the tracer

The tracer became fainter as it was being distributed to other tissue and organs.

Blood pool

Figure 4.2: Blood pool anterior (right) and posterior (left) pelvis static images

The anterior and posterior pelvic region showed accumulation of tracer in the kidneys (Figure 4.2). The tracer's predominant route of clearance (renal) was clearly seen.

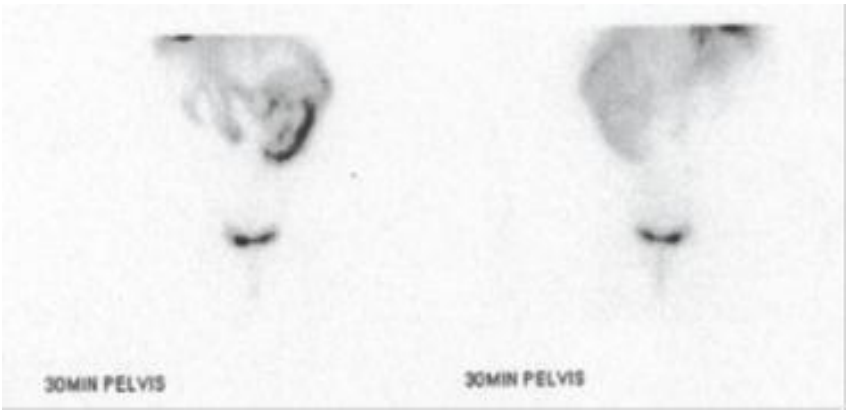


Figure 4.3: Pelvis static images anterior (left) and posterior (right), 30 min post-injection

^{99m}Tc-EC-MN is rapidly cleared renally. The tracer was seen accumulating in the bladder (Figure 4.3). The patient was previously asked to void so that pelvic/tumour localisation would not be confused with its accumulation in urine.

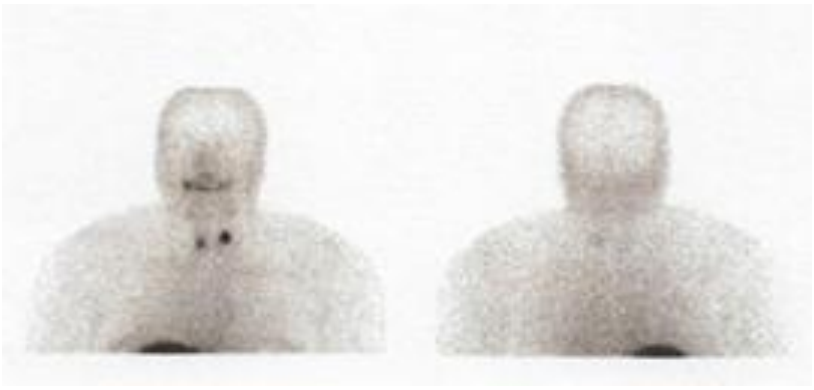


Figure 4.4: Thyroid static, 30 min post-injection. Tracer localised in thyroid of anterior image (left)

The uptake in the thyroid (Figure 4.4) appears non-uniform. This may be an artefact as the neck was not hyper extended and the bony and soft tissues of the jaw may have caused attenuations. Ionic ^{99m}Tc is known for its high affinity for the thyroid. Saha, (2004) states that ^{99m}Tc-chelates are promptly eliminated and they do not accumulate in the thyroid and the choroid plexus of the brain, however, because the radioisotope binding was below 95% the tracer may have dissociated and the free pertechnetate evidently appeared in the thyroid and salivary gland.

SPECT

Figure 4.5 showed that there were no abnormal foci demonstrated on the pelvis. The little warm spots observed were due to accumulation of the tracer in the bladder which is consistent with the clearance of metronidazole.

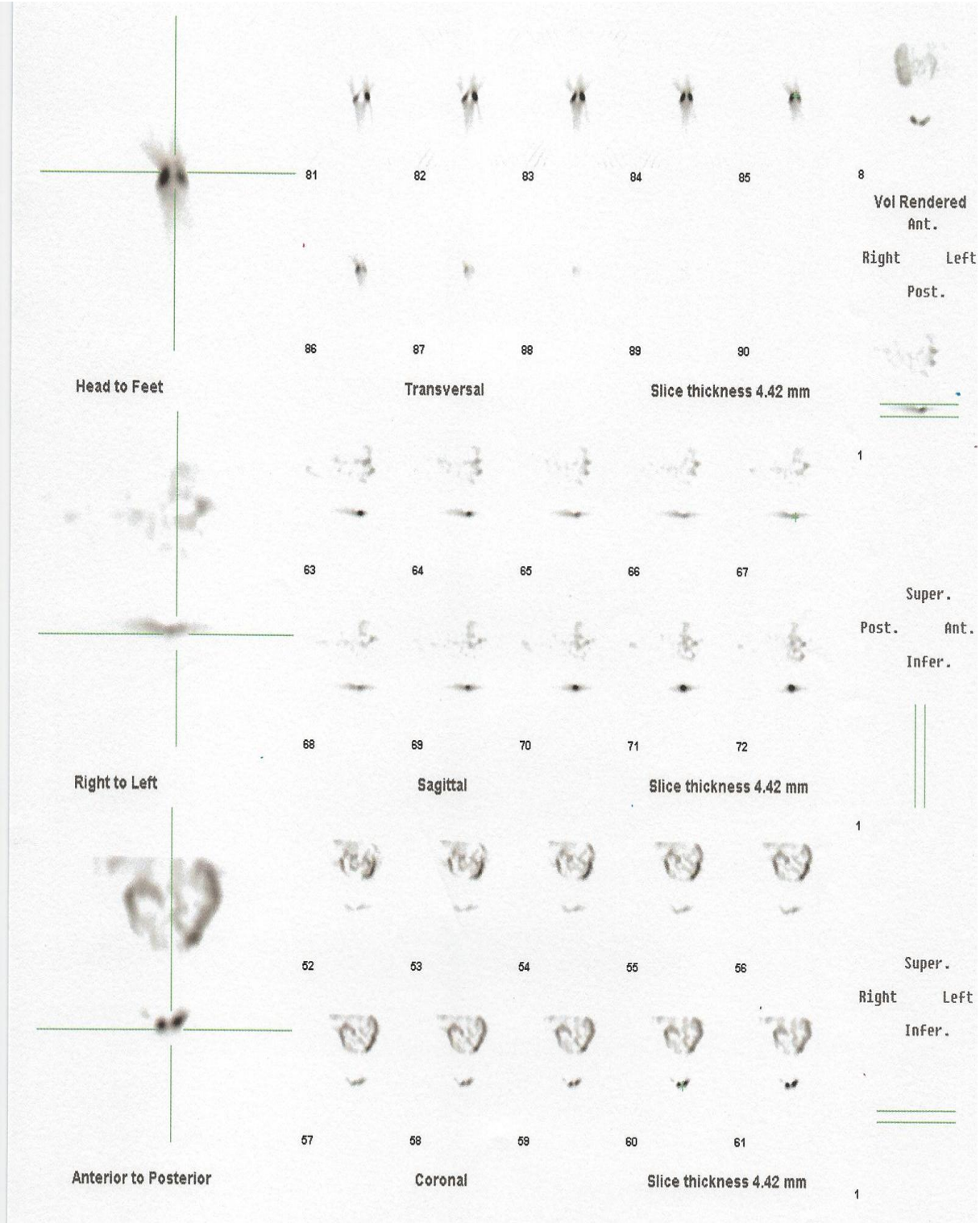


Figure 4.5: SPECT images show no significant or abnormal tracer uptake in the pelvis.

Whole body scans

Scans were interpreted under the guidance of three Nuclear Medicine specialists (Prof. M. Sathekge, Dr. N. Soni and Dr. A.A. Gutta).

In vivo tissue distribution studies from the literature show that ^{99m}Tc-EC-MN localises in the thyroid, intestine, muscle, kidneys, stomach, liver, lung and blood in varying quantities (Yang *et al*., 1999). The scan from Patient 1 (Figure 4.6) was consistent with normal bio-distribution of ^{99m}Tc-EC-MN when compared to Yang *et al.* (1999). Tracer uptake was visible in the salivary gland, thyroid, liver, kidneys, intestine and urinary bladder. This pattern is to be expected as metronidazole is chiefly metabolised in the liver and in the intestine, and excreted in urine. No abnormal accumulation in the cervix or pelvic area was observed; instead there was tracer accumulation in the bladder, which was not visualised after voiding the bladder.

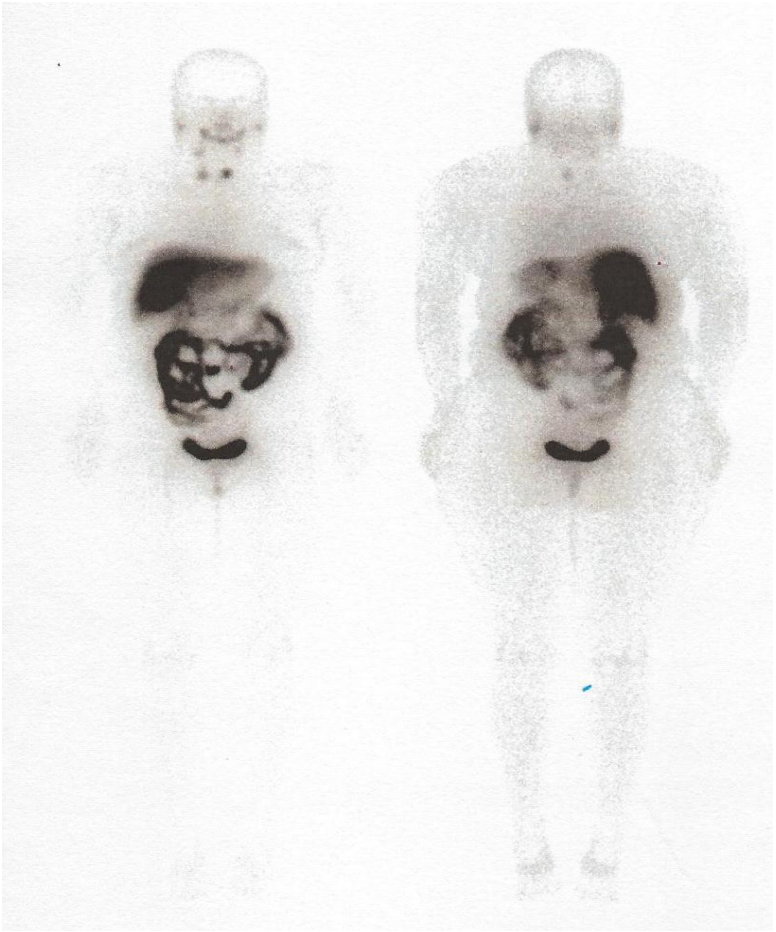
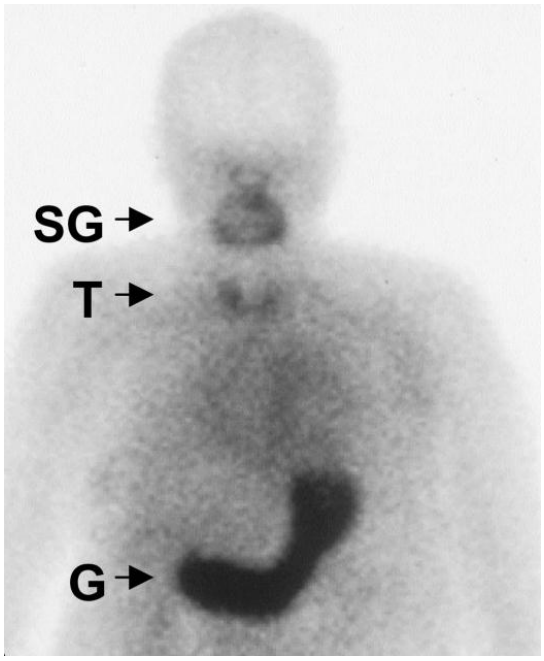


Figure 4.6: Patient 1: Whole body static images. Left = anterior. Right = posterior



Source: Altorjay *et al.*, 2007

Figure 4.7: Uptake of pertechnetate in body organs

Pertechnetate has been widely used for thyroid imaging and other studies. It is documented that its bio-distribution follows a similar pattern to that of radioactive iodine as pertechnetate has been shown to accumulate in organs that have the sodium/iodide symporter (Altorjay *et al.*, 2007). These organs include the salivary and thyroid glands, and the stomach. Figure 4.7 showed visible uptake (hot spots in black) of pertechnetate in the salivary glands (SG), thyroid (T) and the stomach (G).In Figure 4.6 (Patient 1) no uptake of pertechnetate was seen in the liver, the intestines and the kidneys suggesting that it was not pertechnetate that was detected but ^{99m}Tc-EC-MN detected in the scan.

4.3.2.4 Conclusion

The labelling efficiency of the injected tracer was evidently below 95% although the imaged distribution paralleled that of ^{99m}Tc-EC-MN reported by Yang *et al*. 1999. When the images in Figure 4.6 were considered together with the results of DAD and chromatograms of the apparent ^{99m}Tc-EC-MN, the evidence indicates that EC-MN was indeed successfully labelled with pertechnetate. Figure 4.6 indicates that the tracer (^{99m}TC-EC-MN) was visible in the liver and intestines, in contrast to Figure 4.7 where free pertechnetate is seen in thyroid and stomach.

However, Figure 4.6 does not show scintigraphic evidence of abnormal tracer accumulation in the pelvis, which would have suggested hypoxic tissue in the case of a ^{99m}Tc-EC-MN image.

Technically the spatial resolution of SPECT is unable to detect foci below 6-8 mm (Gutta, 2011). The size of the any hypoxic tissue within the tumour may have been very small and SPECT may have been unable to detect it, so there was a high possibility of a false negative. However, there is also a possibility that the cancer may have been free of hypoxia. A positive outcome may therefore be anticipated post-operatively and beyond as the cancer was re-sected

The histological findings did not reveal evidence of tissue hypoxia as no lymphovascular invasion was noted. The absence of VEGF and MVD analytical report leads to an inconclusive investigation.

4.4 SUMMARY

This chapter described the clinical data for the only patient to whom it was possible to administer ^{99m}Tc-EC-MN. The scintigraphic scans showed an uptake pattern that was indicative of successful labelling of EC-MN with ^{99m}Tc. No hypoxic uterine tissue was evident. Clinical markers for tissue hypoxia were unavailable, hence a match between the scans and the clinical data, though apparent, could not be proven.

CHAPTER 5
SUMMARY DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 INTRODUCTION

The findings presented in Chapters 3 and 4 will be summarised and discussed in this chapter. The chapter also describes the limitations of the study, followed by the conclusions and recommendations.

5.2 SUMMARY DISCUSSION

5.2.1 Study Background

Tumour formation is a pathological process in which hypoxia plays a significant role. Hypoxia has been implicated as a major cause of tumour radio- and chemo-resistance, progress and metastasis.

There has been considerable success in identifying groups of hypoxia tracers to detect tumours which may show radio-resistance. One of the groups is the nitro-imidazole compounds. These compounds are trapped in viable hypoxic tissue after being enzymatically reduced (Chu *et al*., 2004). Technetium radiolabelled metronidazole (^{99m}Tc-EC-MN) has the advantage of producing scintillation mages with high tumour to background ratio in a short amount of time (Yang *et al*., 1999).

^{99m}Technetium as the radioisotope of choice has attractive physical properties as well as low price and easy availability when compared with ^{18}F (Yang *et al*., 1999). ^{99m}Technetium possesses additional properties which give it an advantage over other radioisotopes, these include emitting gamma rays between 100-250 KeV, a short half-life that allows for practicality, offers a high target /background ratio for good image contrast and the ability to bind with a variety of ligands and remain stable *in vivo* (University of Limpopo (Medunsa Campus), 2009).

Although ^{99m}Tc-EC-MN has recently found favour in the assessment of cerebrovascular accidents, myocardial infarction and various tumours (Yang *et al*., 1999), the use of direct-acting Eppindorf oxygen-sensitive electrodes remains the gold standard for measuring tissue hypoxia. The latter technique however has major drawbacks such as its invasiveness and its

link with negative effects in cervical cancer patients who undergo radiotherapy. It is also a tedious technique and expensive; which has led to its exclusion as a routine clinical tool for hypoxia detection (Höckel *et al*., 1993; Höckel *et al*., 1996).

5.2.2 Objectives

This study aimed to investigate the use of ^{99m}Tc-EC-MN to determine the degree of hypoxia in cervical cancer. The original study design was to determine whether SPECT with ^{99m}Tc-EC-MN would detect hypoxic cervical cancer lesions and compare the results with the histological report. The practice of safe handling radiopharmaceuticals and gaining knowledge in conducting research formed part of the secondary objective of the study.

5.2.3 Method development

The protocol for the study was pre-approved by the University of Pretoria Research and Ethics Committee in 2006. It was then edited and adapted for an MSc submission to Medunsa Research and Ethics Committee in 2008.

The initial plan was to perform quality control tests (chromatography) on the pertechnetate and on the radiolabelled EC-MN, then administer it to selected patients as per objectives in Section 1.5.

Logistical delays between involved departments at UP/SBAH led to a very late commencement of the study in November 2010. This delay meant that the product had been in refrigerated storage for more than two years. There was no expiry date on the product; hence the stability of the product was an unknown factor at the start of the study. The stability of the product and its suitability for patient administration had to be determined prior to administration. Hence a series of quality control tests for radiochemical purity and labelling efficiency were implemented.

There were originally six sealed, refrigerated vials of EC-MN available for the study. Determination of radiochemical purity of the vials of EC-MN proved to be challenging. There was also, an older batch of EC-MN in Eppindorf tubes which had expired in 2007. This product was compared chromatographically with the more recent batch for radiochemical purity and labelling efficiency. **Hence the focus of the study changed from a clinical emphasis to a more chemistry-based study.**

The radiopharmaceutical quality control studies and the SPECT images were conducted in the Nuclear Medicine department at SBAH. The HPLC analyses were conducted at NTP Radioisotopes (Pty) Ltd.

The chronological sequence of events for the quality assurance method development is summarised below. The details of each step are in found in the sub-sections that are listed in parentheses:

- *Solubilisation of tin*: The tin (II) chloride ($SnCl_2$) must be solubilised in order to reduce pertechnetate ($^{99m}TcO^-_4$) to $^{99m}TcO^{3+}$ (+5 oxidation state) (3.4.3).

- *Radiolabelling EC-MN*: To form a radioactive tracer (3.4.4).

- *^{99m}Tc-EC-MN chromatography I*: The stability of the EC-MN was not known. Stability affects labelling efficiency; hence the radiochemical purity of the radiolabelled product had to be established (3.4.5).

- *Radiolabelling detection with the ionisation chamber*: To measure the activity at the origin and solvent front and calculate the labelling yields thereafter (3.4.6).

- *^{99m}Tc-colloid chromatography*: To determine the efficiency of pertechnetate reduction by $SnCl_2$ by reducing a pertechnetate solution in the absence of EC-MN, thereby determining the resultant ^{99m}Tc-colloid and free pertechnetate as an indication of efficiency of the reduction reaction (3.4.7).

- *^{99m}Tc-EC-MN chromatography II*: Compare the Rf value of ^{99m}Tc-EC-MN with pertechnetate and ^{99m}Tc-colloid thereby determining whether the EC-MN had been radiolabelled successfully with $^{99m}TcO_4$ (3.4.8).

- *Pertechnetate chromatography I*: The Rf value of ^{99m}Tc-EC-MN was unknown. It was therefore necessary to determine the Rf for free pertechnetate, a by-product of poor labelling and Rf of ^{99m}Tc-EC-MN. The results were then used as a standard of comparison (3.4.9).

- *Pertechnetate chromatography II*: To determine whether the size of the chromatography “spots” or drops, affected the shape, size or position of the peaks of the scans (3.4.10).

- *Determination of labelling efficiency and stability of expired EC-MN*: For future comparison of stability and labelling efficiency with the unexpired batch (3.4.11).

- *Determination of labelling efficiency of vials of EC-MN*: Comparison of stability and labelling efficiency with the expired batch (3.4.12).

- *Labelling EC-MN for patient dose*: To use as a tracer for hypoxic cervical cancer cells (3.4.13).

5.2.3.1 Solubilisation of tin (II) chloride

Saha (2004) and Kneen, Rogers and Simpson (1971) state that tin (II) chloride is hydrolysed in water, so there were concerns over the practical feasibility of dissolving tin (II) chloride water as it forms insoluble hydrolysed tin compounds, such as chlorohydroxo tin (II) indicated in the equations below.

$6\ SnCl_2\ (aq) + O_2\ (g) + 2\ H_2O\ (l) \rightarrow 2\ SnCl_4\ (aq) + 4\ Sn\ (OH)\ Cl\ (s)$

$SnCl_2 + H_2O \rightarrow Sn\ (OH)Cl\ (s) + HCl$

Note: (s) = insoluble solid

(aq) = water-soluble

The results showed that tin (II) chloride did not dissolve in 1 ml water. Instead a cloudy solution with precipitate was formed. Tin (II) chloride is known to dissolve in HCl (Saha, 2004) that approach was followed but a precipitate formed when the pH was adjusted above pH 2. Thus the HCl method was not an option.

The dissolution of tin (II) chloride in water was repeated, but the 9 ml sterile water was added rapidly to the tin (II) chloride in one aliquot and shaken vigorously to produce a clear solution of solubilised tin (II) chloride.

5.2.3.2 Radiolabelling EC-MN

EC-MN was dissolved in 0.3 ml sterile water, and then 0.1 ml tin (II) chloride solution was added. Pertechnetate was mixed with the latter and made up to 1 ml with sterile water. A clear pale straw-coloured solution of ^{99m}Tc-labelled metronidazole resulted. The solution was then filtered through a 0.22 µm filter into a sterile vial and the radiolabelling efficiency was assessed.

5.2.3.3 ^{99m}Tc-EC-MN chromatography I

The purpose of chromatography was to assess whether EC-MN was indeed labelled with pertechnetate as in the method above (see Section 3.4.4 and 5.2.3.2.)

The results below (Table 5.1) show that acetone and ethyl acetate-developed chromatograms had features of poor separation, whereas ethanol- and saline-developed chromatograms had single peaks, thus better separation. However, the peaks remained unidentifiable (^{99m}Tc-colloid, $^{99m}TcO_4^-$ or ^{99m}Tc-EC-MN), although the quality of the scans

increased with increased eluting power of solvents (see Table 2.3). Therefore a ^{99m}Tc-colloid study was performed.

Table 5.1: Summary results of ^{99m}Tc-EC-MN chromatography

Mobile phase	Stationary phase	Summary results
		Chromatography I (see Section 3.4.5)
Acetone	ITLC-SG	- multiple peaks with broad peak at solvent front - ^{99m}Tc-colloid or residual activity at origin?
Ethyl acetate	ITLC-SG	-large peak at origin (^{99m}Tc-EC-MN of free $^{99m}TcO_4$?) -high residual activity at solvent front
Ethanol	ITLC-SG	-large peak at solvent front -no ^{99m}Tc-Colloid at origin -less residual activity than acetone ethyl acetate developed scans
Saline	ITLC-SG	-clean peak at solvent front -less residual activity than ethanol developed scan -no ^{99m}Tc-colloid at origin
		Chromatography II (see Section 3.4.8)
Acetone	ITLC-SG	-no difference from chromatography I -high residual activity
Ethyl acetate	ITLC-SG	-similar to chromatography I -high residual activity
Ethanol	ITLC-SG	-low residual activity -better defined peak at solvent front than that in chromatography I -no ^{99m}Tc-colloid at origin
Saline	ITLC-SG	-low residual activity -no peak at origin, therefore no ^{99m}Tc-colloid -peak at solvent front had brad base

5.2.3.4 Radiolabelling detection with ionisation chamber

ITLC was performed in the same manner as above (Table 5.1), except that activity on the strips was measured by the 'cut and count' method and the ionisation chamber. The strips were initially cut in half separating the origin from the solvent front and radioactivity counts were measured separately.

Results showed a labelling yield below 95% on all chromatograms. These measurements were however inconclusive as residual activity remaining on the chromatogram distorted the results.

5.2.3.5 ^{99m}Tc-colloid chromatography I

^{99m}Tc-colloid is a by-product of inefficient labelling and usually separates from the sample under investigation. Chromatography was performed to assess the ability of the tin (II) chloride solution to reduce pertechnetate quantitatively in the absence of EC-MN. The location of the reduced form of pertechnetate peak (^{99m}Tc-colloid) remained at Rf 0.

Results showed a distinct peak of ^{99m}Tc-colloid on the origin with no free pertechnetate at solvent front with both ethanol- and saline-developed chromatograms.

5.2.3.6 ^{99m}Tc-EC-MN chromatography II

The chromatography of ^{99m}Tc-EC-MN was repeated the following day to confirm results (see Table 5.1) for summarised results). The Rf value of ^{99m}Tc-EC-MN when developed in saline and ethanol appeared very close to that of pertechnetate as documented in literature (Rf=1) (Theobald, 2011).

5.2.3.7 Pertechnetate chromatography II

Acetone-developed pertechnetate chromatograms consistently showed irregular peaks (Figures 3.11 and 3.12). This was a concern because acetone is endorsed by the International Pharmacopoeia (W.H.O., 2008) for the chromatography of pertechnetate. However, the syringe technique of “spotting” pertechnetate on the ITLC strips may have varied, producing a lack of control of droplet size. The technique was adapted by using a micropipette to optimise control of the spot size.

Firstly a 2 µl droplet of pertechnetate was “spotted” on each 120 mm ITLC-SG strip, using a micropipette. Secondly the same droplet size was spotted on 200 mm ITLC-SG strips. Ethanol, saline and acetone were the solvents used. Finally a 5 µl droplet was spotted on acetone-developed chromatograms to determine whether droplet size affected the shape, size and position of the peak.

The results showed that a large droplet (5 µl) results in distinct separation of components within the compound in acetone–developed chromatograms (see Figure 3.19). Saline and ethanol chromatograms (see Figure 3.20-3.23) consistently showed single peaks, leading to their selection as solvents of choice further analysis.

5.2.3.8 Determination of labelling efficiency and stability of expired EC-MN

HPLC coupled to a Gabi Star Ray test gamma detector (radioactivity detector) and a Diode Array Detector (DAD) was used to clarify efficiency of EC-MN labelling with pertechnetate. The expired ^{99m}Tc-EC-MN product was used as a control to determine the stability of ^{99m}Tc-EC-MN intended for patient use by comparison.

ITLC results demonstrated that ethanol-developed chromatograms (Figures 3.26 and 3.27) had poor labelling of EC-MN irrespective of doubling tin (II) chloride to improve pertechnetate binding to the ligand. In the saline-developed chromatogram in Figure 3.24 the peak at the solvent front indicates that there was some degree of binding or it wasfree pertechnetate. As the tin (II) chloride concentration was increased in Figure 3.25 the poor labelling became evident as demonstrated by the large peak at the origin. Overall, the expired ^{99m}Tc-EC-MN had poor labelling characteristics. HPLC techniques were used to clarify whether the compound shown at the solvent front in Figure 3.24 of the saline-developed chromatograms, was free pertechnetate or labelled EC-MN.

The HPLC results indicate that EC-MN was not bound to pertechnetate, as the retention times of ^{99m}Tc-EC-MN (see Figure 3.30 and 3.31) were the same as that of pertechnetate (see Figure 3.32). Increasing tin (II) chloride concentration did not improve the labelling either.

5.2.3.9 Determination of labelling efficiency of vials of EC-MN

The ethanol chromatogram (Figure 3.33: bottom) produced a peak at the origin which was consistent with ^{99m}Tc-colloid in Figure 3.25 of the saline-developed chromatograms. However, a comparison of all the ethanol-developed chromatograms with those that were developed in saline suggests that ^{99m}Tc-EC-MN is carried to the solvent front with both solvents; Figure 3.33 was the exception, which means that the peak observed at the origin may have been residual activity.

Results from HPLC coupled with the DAD (Figure 3.34) showed a compound at a different retention time (approximately 6.5 minutes) from that which appeared in Figure 3.28 and 3.29 of expired ^{99m}Tc-EC-MN (retention times were approx. 10minutes). These times indicated that the compound was not pertechnetate.

Further analysis with the radioactivity detector also showed that there was a labelled compound other than pertechnetate. Clarification was offered by comparison of the peak observed in Figure 3.35 of vial ^{99m}Tc-EC-MN detected with the radioactivity detector, with that of pertechnetate in Figure 3.32 which evidently indicated that Figure 3.35 shows

successfully labelled EC-MN. It also indicated that it was safe to prepare the remaining vials for administration to patients.

5.2.3.10 Chromatography of ^{99m}Tc-EC-MN for Patient 1

Chromatography was performed on ^{99m}Tc-labelled EC-MN with a Veenstra chromatogram scanner (VCS 201). A single peak was seen at the solvent front which was, by implication, the labelled product. This chromatographic scan indicated successful labelling, hence the product was administered to a patient (Patient 1). The degree of labelling could not be established but it was below 95% and differed slightly from the product shown in one of the developer's scans from pilot data (Figure 3.38) which had labelling efficiency greater than 95%.

The differences between the scans obtained in this study and the developer's scans may have been due to the following reasons:

- ITLC-SG was used in this study versus ITLC-SG "plates" (unspecified) that were used by the developers
- A Veenstra chromatogram scanner (VCS 201 Veenstra-instruments, Netherlands) was used verses a radio-TLC scanner (Bioscan, Washington, DC) used by the developer.

The administration to the patient was approved by the HOD of the Nuclear Medicine department

5.2.3.11 Chromatography of ^{99m}Tc-EC-MN for Patient 2

^{99m}Tc-EC-MN prepared for Patient 2 would not pass through the 0.22 µm sterile filter. Comparison with ^{99m}Tc-EC-MN of Patient 1 suggested insoluble compounds had formed in the labelling process of the EC-MN for Patient 2 The product was hence unsuitable for administration and this part of the study was therefore, unfortunately aborted.

5.2.4 Clinical Application

Due to logistical constraints explained in the introduction of Chapter 3, only two of the original vials of product were left to administer to patients. As described above, the product produced from the second vial was not suitable for administration to the second patient.

5.2.4.1 Clinical data for Patient 1

Patient History

Patient 1, referred from Rob Ferreira Hospital, was diagnosed with stage IIa1 cancer of the uterine cervix. She was booked for surgery the day after imaging.

Investigations

Full blood counts results were normal, which indicated that the oxygen-carrying capacity of blood was not compromised. There was no evidence to suggest obstructive uropathy on ultrasound and no evidence of pulmonary metastases was noted on x-ray. Macroscopic and microscopic evaluation revealed that the resection margins were tumour-free. Three of eight identified pelvic lymph nodes had metastatic tissue, however, no lymphovascular involvement was noted.

Logistical problems lead to the cancellation of the analysis of hypoxia markers (VEGF and MVD) as the examination was not financially feasible. Therefore the presence or the absence of tumour hypoxia could not be confirmed.

Post-surgical intervention

Patient 1 was readmitted and underwent irradiation treatment, brachytherapy and one course of chemotherapy. She was then discharged, after completion of treatment.

5.2.4.2 Tumour Hypoxia Imaging for Patient 1

Patient 1 was administered ^{99m}Tc-EC-MN for detection of possible hypoxia, prior to surgery.

Imaging procedure

30 mCi ^{99m}Tc-EC-MN was injected IV, followed immediately by dynamic images (blood flow and pool) static images (pelvis and thyroid), SPECT and whole body scintigraphy.

Tracer uptake was visible in the renal blood circulation. Blood pool images (Figure 4.2) showed accumulation of tracer in the kidneys, the main route of elimination for pertechnetate and ^{99m}Tc-EC-MN. Pertechnetate is known for its high affinity for the thyroid as seen in Figure 4.4. There was only faint thyroid uptake suggesting that there was a small fraction of free pertechnetate *in vivo.* Tracer uptake was clearly evident in the liver, kidneys and intestines, suggestive of bound ^{99m}Tc-EC-MN and not free pertechnetate (Figure 4.6). Hence labelling was apparently successful.

SPECT and whole body scintigraphy

No scintigraphic evidence of abdominal tracer accumulation was noted in the pelvis in the SPECT images (Figure 4.5). Figure 4.6 of the whole body scintigraphy showed uptake of ^{99m}Tc-EC-MN in the liver, intestines, bladder and little uptake in the salivary and thyroid glands, which parallels the distribution of ^{99m}Tc-EC-MN in the study by Yang *et al.,* (1999). A comparison with the pertechnetate scan shown in Figure 4.7 indicated that ^{99m}Tc-EC-MN was indeed detected in Patient 1, rather than pertechnetate.

The absence of scintigraphic evidence of abnormal tracer accumulation in the pelvis (Figure 4.6), suggested that there was no hypoxic tissue of the tumour. The histological findings also did not reveal evidence of tissue hypoxia. The investigation was rendered inconclusive as the analyses for VEGF and MVD were omitted.

5.3 CHALLENGES AND LIMITATIONS OF THE STUDY

5.3.1 Equipment

Multiple challenges arose.

- There were printer problems with the computer system linked to the IAEA-donated chromatography scanner (Veenstra instruments, VSC 201) such that printouts of the chromatograms could initially not be obtained. This delayed the project as no manuals, supplier details or maintenance records were available.
- No designated appropriately-trained personnel for the above mentioned equipment were available to assist on site.
- There were no records for the calibration of the Veenstra VSC 201 radio-chromatogram scanner. As a result the reliability of the scans could not be established.

5.3.2 EC-MN

- The EC-MN that was intended for the study did not have an expiry date, so its stability could not initially be confirmed.
- EC-MN is undergoing clinical trials and is only approved as such by the FDA; it has not been registered as a drug for general clinical use. It was impossible to access newly formulated vials to be used for the study, from the supplier. Labelling efficiency

and radiochemical purity tests had to be performed on the vials that were available (6).

- Radiochemical assays for labelling efficiency of EC-MN meant that only two vials remained for potential administration to patients.

5.3.3 Measures of hypoxia

- The HoD of Anatomical Pathology Department of UP/SBAH was unavailable due to severe illness (cerebrovascular accident). She unfortunately fell ill before permission was obtained to access the histological evaluation of the tumours and because she could no longer communicate, that objective of the study was no longer pursued. However, when she had recovered, a request to test for VEGF and MVD was made but it was declined because the kit was too expensive to be purchased for only one specimen (Patient 1). As a result, it was difficult to conclude whether ^{99m}Tc-EC-MN was successfully labelled and the tumour was not hypoxic or vice versa.

5.3.4 Communication

- Communication with the product developer was extremely difficult and few responses were obtained to communications and questions.

5.4 CONCLUSIONS

1. Quality Control procedures were applied in the determination of the safety of the radiopharmaceutical, ^{99m}Tc-EC-MN. Aseptic preparation was followed up to the point of administration. The challenges faced assisted the researcher in gaining 'hands-on' experience of conducting and co-managing both clinical- and chemistry-based research.

2. Radiochemically-related-conclusions that were drawn include the following points:

    i. Tin (II) chloride can be solubilised in water – a point which is not well documented in the literature.

    ii. In ITLC-SG chromatography, ^{99m}Tc-EC-MN migrates to the solvent front when developed in ethanol and saline.

iii. The ITLC method provided by the product supplier did not separate ^{99m}Tc-EC-MN from free pertechnetate as they appear at the same Rf.

3. Labelling of EC-MN with pertechnetate ($^{99m}TcO^-_4$) was achieved and confirmed by scintigraphy.

4. Successful labelling of EC-MN with ^{99m}Tc can be achieved up to two years after kit manufacture given appropriate storage conditions for the EC-MN.

5. The results from the case study of Patient 1 indicated that ^{99m}Tc-EC-MN was detected *in vivo* with a tracer distribution that differs from that of pertechnetate and parallels that described by Yang (1999).

6. There was no scintigraphic evidence of hypoxia detected with SPECT in the pelvic area in Patient 1.

7. Morphology of the tumour could not establish the presence of hypoxia. Because analysis with hypoxia markers was not performed, no conclusion could be drawn from the histology report.

8. The hypoxic status of the tumour remained inconclusive; therefore the prognostic impact of ^{99m}TC-EC-MN in cervical cancer was not determined in this study.

5.5 RECOMMENDATIONS

The following recommendations are made based on the results of the study:

5.5.1 EC-MN

- Recommendations regarding product stability and potential expiry should be available for all products, even in the developmental stages and particularly for clinical trials.

5.5.2 Equipment

- The IAEA should insist on appropriate training and quality management processes (maintenance and records) for all donated equipment.
- Manuals, supplier details and service records should be available and current for all departmental equipment.

- A designated, appropriately-trained person should be responsible for the above. .
- Equipment should be calibrated regularly and processes checked with internal standards.
- The IAEA Operational Guidance and Technical Documents (especially Tec Doc 602) on radiopharmacy procedures should be followed for equipment used in a 'hot' laboratory.
- The manufacturing company should re-evaluate the efficacy of the chromatogram scanner. An increased lead thickness of the collimator may improve the results.

5.5.3 Communication and Delegation

- Communication among all the role players in the research is very important.
- Role definition in research is important, particularly when the research includes a multidisciplinary group. A suitably senior study co-ordinator should be appointed at the host institution to work with the researcher in order to facilitate the smooth running of the study.
- Good communication with the supplier/product developer is essential.

5.5.4 Future Research

- A simple Quality Control method to separate ^{99m}Tc-EC-MN from free pertechnetate should be developed.
- Further studies are required in order to confirm the efficacy of ^{99m}Tc-EC-NM in determining tumour hypoxia in cervical cancer. If a suitable animal or *ex vivo* model is not available, patients with known cancer tissue hypoxia should be evaluated and compared with those who are non-hypoxic.
- A study with ^{99m}Tc-EC-MN should be conducted on cervical cancer in patients with early stage hypoxia who are still eligible for surgery, over a period that allows assessment of the outcome in comparison to those predicted by ^{99m}Tc-EC-MN.

5.6 CLOSURE

This study provided a useful vehicle for gaining expertise in the handling and QC of radiopharmaceuticals.

The work has again highlighted the challenges associated with working with tin salts as reducing agents. The production of suitable ‘cold kits’ for radiolabelling in diagnostics and therapy is also a complex process.

Appropriate QC methods for small-scale preparation of radiopharmaceuticals are critical for optimal imaging to occur.

The study has demonstrated that ^{99m}Tc-EC-NM can be prepared in a hospital setting, though its usefulness in the detection of hypoxic areas of cervical cancer was not able to be determined.

REFERENCES

ALTORJAY, A., DOHĀN, O., SZILĀGYI, A., PARODER, M., WAPNIR, I.L. & CARRASCO, N. 2007. Expression of the Na^+/I^- symporter (NIS) is markedly decreased or absent in gastric cancer and intestinal metastatic mucosa of Barrett oesophagus. *BMC Cancer.* 7:5.

AHFS see AMERICAN HOSPITAL FORMULARY SERVICE

AMERICAN HOSPITAL FORMULARY SERVICE. 2008. *Drug Information: The most comprehensive authoritative source of evaluative drug information.* Bethesda: American Society of Health-system. Pharmacists Inc.

BACHTIARY, B., SCHINDL, M., PÖTTER, R., KNOCKE, T.H., HAINFELLNER, J.A., HORVAT, R., DREIER, B. & BIRNER, P. 2003. Overexpression of hypoxia-inducible factor 1 indicates diminished response to radiotherapy and unfavourable prognosis in patients receiving radical radiotherapy for cervical cancer. *Clin Cancer Res*. 9:2234-2240.

BECK, I., RAMIREZ, S., WEINMANN, R. & CARO, J. 1991. Enhancer element at the 3`-flanking region controls transcriptional response to hypoxia in the human erythropoietin gene. *J Biol Chem*. 266:15 563-15 566.

BEYER, T., TOWNSEND, D.W. & BLODGETT, T.M. 2002. Dual-modality PET/CT tomography for clinical oncology. *Q J Nucl Med*. 46(1):24-34.

BOSCH, F.X., MANOS, M.M., MUNOZ, N., SHERMAN, M., JANSEN, A.M., PETO, J., SCHIFFMAN, M.H., MORENO, V., KURMAN, R. & SHAH, K.V. 1995. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. *F Natl Inst*. 87:796-802.

BRIZEL, D.M., SCULLY, S.P., HARRELSON, J.M., LAYFIELD, L.J., BEAN, J.M., PROSNITZ, L.R. & DEWHIRST, M.W. 1996. Tumour oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. *Cancer Res*. 56:941-943.

BROWN, J.M. 1999. The hypoxic cell: A target for selective cancer therapy. Eighteenth Bruce F. Cain Memorial Award Lecture. *Cancer Res*. 59:5863–5870.

CANDELARIA, M., GARCIA-ARIAS, A., CETINA, L. & DUENAS-GONZALEZ, A. 2006. Radiosensitisers in cervical cancer. Cisplatin and beyond. *Radiat Oncol*. 1:15.

CHICHAREON, S., HERRERO, R., MÜNOZ, N., BOSCH, F.X., JACOBS, M.V., DEACON, J., SANTAMARIA, M., CHONGSUVIVATWONG, V., MEIJER, C.J. & WALBOOMERS, J.M. 1998. Risk factors for cervical cancer in Thailand: a case-control study. *F Natl Cancer Inst*. 90:50-56.

CLIFFORD, R. 2011, April 6. Triphasic Bone Scan In: *Wheeless' Textbook of orthopaedics.* [Online]. Avaliable at: <http://www.wheelessonlline.com/ortho/triphasic_bone_scan>.Accessed: 01/09/2011.

COMERCI, G., BOLGER, B.S., FLANNELLY, G., DE BARROS LOPES, A. & MONAGHAN J.M. 1998. Prognostic factors in surgically treated stage Ib-IIb carcinoma of the cervix with negative lymph nodes. *Int J Gynecol Can*. 8:23-26.

CHU, T., LI, R., HU, S., LIU, X. & WANG, X. 2004. Preparation and biodistribution of Tc-99m-labelled 1-(2-nitromidazole-1-yyl)-propanhydroxyiminoamide (N2IPA) as a tumour hypoxia maker. *Nucl Med Biol*. 3:199-203.

DALY, S.F., DOYLE, M., ENGLISH, J., TURNER. M., CLINCH, J. & PRENDIVILLE, W. 1998. Can the number of cigarettes smoked predict high-grade cervical intraepithelial neoplasia among women with mildly abnormal cervical smears? *Am J Obstet Gynecol*. 179: 399-402.

DEHDASHTI, F., GRIGSBY, P.W., MINTUN, M.A., LEWIS, J.S., SIEGEL, B.A. & WELCH, M.J. 2003. Assessing tumor hypoxia in cervical cancer by positron emission tomography with 60Cu-ATSM: relationship to therapeutic response- a preliminary report. *Int J Radiat Oncol Biol Phys*. 55(5):1233-1238.

DENNY, L. 2010. Cancer of the cervix. [Online]. April 9. Available at: <http://www.health24.com/medical/Condition_centres/777-792-4101-4102,29416.asp> Accessed: 26/10/2010.

DENTHRE COLLEGE. Standardbase techniques: thin layer chromatography. [Online]. 2010. The Netherlands. Available at: <http://www.standardbase.hu/tech/Thin%20Layer%20Chromatography.pdf> Accessed: 23/11/2010.

DEPARTMENT OF HEALTH. Not dated. National guideline on cervical cancer programme. Pretoria. Available at: <http://www.doh.gov.za/docs/factsheets/guidelines/cancer.pdf> Accessed: 22/01/2010.

DoH see DEPARTMENT OF HEALTH

DUEÑAS-GONZALEZ, A., CETINA, L., MARISCAL, L. & DE LA GRAZA, J. 2003. Modern management of locally advanced cervical carcinoma. *Cancer Treat Rev*. 29:389-399.

ELSING, P., TODDE, S., PENUELA, I., MEYER, G., FARSTAD, B., FAIVRE-CHAUVET, A., MIKOLAJCZAK, R., WESTERA, G., GMEINER-STOPAR, T., DECRISTOFORO, C. & THE RADIOPHARMACY COMMITTEE OF THE EANM. 2010. Guidance on current good radiopharmacy practice (cGRPP) for the small-scale preparation of radiopharmaceuticals. *Eur J Nucl Med Imaging.* 37:1049-1062.

FIFIELD, F.W. & KEALEY, D. 1995. *Principles and practice of analytical chemistry*. 4th ed. London: Chapman & Hall.

FYLES, A.W., MILOSEVIC, M., PINTILIE, M. & HILL M. 1998. Cervix cancer oxygenation measured following external radiation therapy. *Int J Radiat Oncol Biol Phys*. 42:751-753.

GERSHENSON, D.M. 2005. Fertility-sparing surgery for malignancies in women. *J Natl Cancer Inst Monogr*. 34:43-47.

GIATROMANOLAKI, A. & HARRIS, A.L. 2001. Tumour hypoxia, hypoxia signalling pathways and hypoxia inducible factor expression in human cancer. *Anticancer Res*. 21:4317-4324.

GIGLIO, J., FERNÁNDEZ, S., REY, A. & CERECETTO, H. 2011. Synthetic and biological characterisation of novel dithiocarbamate containing 5-nitromimidazole ^{99m}Tc-complexes as potential agents for targeting hypoxia. *Bioorg Med Chem Lett.* 21(1): 394-7GRAY, L.H., CONGER, A.D. & EBERT, M. 1953. The concentration of oxygen dissolved in tissues at the time of irradiation as factor in radiotherapy. *Br J Radiol*. 26:683.

GREEN, J.A., KIRWAN, J.M. & TIERNEY, J.F. 2001. Survival and recurrence after concomitant chemotherapy and radiotherapy for cancer of the uterine cervix: a systematic review and meta-analysis. *Lancet*. 355:781-786.

GRIGSBY, P.W., DEHDASHTI, F. & SIEGAL, B.A. 1999. FDG-PET evaluation of carcinoma of the cervix. *Clin Positron Imaging*. 2(2):105-109.

GRIGSBY, P.W., PEREZ, C.A., CHAO, K.S.C., HERZOG, T., MUTCH, D. & RADER, J. 2001. Radiation therapy for carcinoma of the cervix with positive para-aortic lymph nodes. *Int J Radiat Oncol Biol Phys*. 49:733-738.

GRIGSBY, P.W., WATSON, M., POWELL, M.A., ZHANG, Z. & RADER, J.S. 2006. Gene expression patterns in advanced human cervical cancer. *Int J Gynecol Cancer*. 16:562-567.

GRISARU, D., ALMOG, B., LEVINE, C., METSER, U., FISHMAN, A., LERMAN, H., LESSING, J.B. & EVEN-SAPIR, E. 2004. The diagnostic accuracy of 18F-flourodeoxyglucose PET/CT in patients with gynaecological malignancies. *Gynecol Oncol*. 94(3):680-684.

GUTTA, A.A., 2011. Personal communication from Dr. A.A. Gutta, Department of Nuclear Medicine. University of Limpopo (Medunsa Campus). 27 July.

HANAHAN, D. & FOLKMAN, J. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 86:353-364.

HRICAK, H. & YU, K.K. 1996. Radiology in invasive cervical cancer *Am J of Roent.* 167:1101-1108.

HÖCKEL M., KNOOP C., SCHLENGER, B., VORNDRAN, B., BAUBNANN, E., MITZE, M., KNAPSTEIN, P.G. & VAUPEL, P. 1993. Intra-tumoral pO_2 predicts survival in advanced cancer of the uterine cervix. *Radiother Oncol*. 26:45-50.

HÖCKEL, M., SCHLENGER, K., ARAL, B., MITZE, M., SCHÄFFER, U. & VAUPEL, P. 1996. Association between tumour hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res.* 56:4509-4515.

HÖCKEL, M., SCHLENGER, K., HÖCKEL, S., ARAL, B., SCHÄFFER, U. & VAUPEL, P. 1998. Tumour hypoxia in pelvic recurrences of cervical cancer. *Int J Cancer*. 79:365-369.

IAEA NUCLEUS. 2010. Elution of the 99Mo→99mTc generator. Internationa Atomic Energy Agency [Online] Available at <http://nucleus.iaea.org/HHW/Radiopharmacy/VirRad/Eluting_the_Generator/Generator_Module/Elution_of_the_99Mo99mTc_generator/index.html>. Accessed 12/11/2011.

IYER, N.V., KOTCH, L.E., AGANI, F., LEUNG, S.W., LAUGHNER, E., ROLAND, H., WENGER, R.H., GASSMANN, M., GEARHART, J.D., LAWLER, A.M., YU, A.Y. & SEMENZA, G.L. 1998. Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1A. *Genes Dev*. 12:149-162.

JEMAL, A., THOMAS, A., MURRAY, T. & THUN, M. 2002. Cancer statistics, 2002.*Cancer J Clin*. 52:23-47.

KERBEL, R.S. 2000. Tumour angiogenesis: past, present, and the near future. *Carcinogenesis*. 21:505-515.

KIM, E.E. 2004. Whole-body positron emission tomography and positron emission tomography/computed tomography in gynaecologic oncology. *Int J Gynecol Cancer*. 14(1):12-22.

KJELLBERG, L., HALLMANS, G., AHREN, A.M., JOHANSSON, R., BERGMAN, F., WADELL, G., ANGSTROM, T. & DILLNER, J. 2000. Smoking, diet, pregnancy and oral contraceptive use as risk factors for cervical intra-epithelial neoplasia in relation to human papillomavirus infection. *Br J Cancer*. 82:1332-1338.

KNEEN, W.R., ROGERS, M.J.W. & SIMPSON, P. 1971. *Chemistry: facts, patterns and principles*, London: Addison Wesley Publishers Ltd.

KNOESEN, O. 2011. Personal communication from Dr. O. Knoesen. Radiochemist. NTP Radioisotopes (Pty) Ltd. 1 June.

KOCH, R.L., BEAULIEU, B.B. Jr., CHRYSTAL, E.J. & GOLDMAN, P. 1981. A metronidazole metabolite in human. Urine and its risk. *Science.* 211(4480):398-400.

KOH, W-J., RASEY, J.S. & EVANS, M.L. 1992. Imaging of human tumours with [F-18] fluoromisonidazole. *Int J Radiat Oncol Biol Phys*. 22:199-212.

KRCAL, A., MITTERHAUSER, M., LETTER, K., DUDCZAK, R. & PIRICH. C. 2002. Comparison of Carrier-Added [99mTc] EDTMP and Carrier-Free Preparations of [99mTc] EDTMP and [99mTc] DPD. In: Radioactive Isotopes in Clinical Medicine and Research. 25th International Symposium, Bad Gastein Austria: *Eur J Nucl Med Mol Imag.* 29(3):S1-S24.

LANDONI, F., MANEO, A., COLOMBO, A., PLACA, F., MILANI, P., PEREGO, P., FAVINI, G., FERRI, L. & MANGIONI, C. 1997. Randomized study of radical surgery versus radiotherapy for stage IB-IIA cervical cancer. *Lancet*. 350:535–540.

LEWIS, J.S., MCCARTHY, D.W., MCCARTHY, T.J., FUJIBAYASHI, Y. & WELCH, M.J. 1999. Evaluation of ^{64}Cu-ATSM *in vitro* and *in vivo* in a hypoxic tumor model. J Nucl Med. 40:177-183.

MAKWELA, R. 2010. Personal communication from Dr. R. Makwela. Consultant. Department of Gynaecological Oncology. University of Pretoria. 12 July.

MALLIA, M.B., MATHUR, A., SUBRAMANIAN, S., BANERJEE, S., SARMA, H.D. & VENKATESH, M. 2005. A novel $[^{99m}Tc{\equiv}N]^{2+}$ complex of metronidazole xanthate as a potential agent for targeting hypoxia. *Bioorganic & Medicinal Chemistry Letters.* 15:3398-3401.

MAYR, N.A., TALI, E.T., YUY, W.T., BROWN, B.P., WEN, B.C., BULLER, R.E., ANDERSON, B. & HUSSEY, D.H. 1993. Cervical cancer: application of MR imaging in radiation therapy. *Rad*. 189:601-608.

MILLER, T.R. & GRIGSBY, P.W. 2002. Measurement of tumour volume by PET to evaluate prognosis in patients with advanced cervical cancer treated by radiation therapy. *Int J Radiat Oncol Biol Phys*. 53:353-539.

MOLHERE, V.M. 2008. Radical Trachelectomy: A fertility-sparing option for invasive cervical cancer. OncoLog. [Online]. 53(6), June. Available at: <http://www2.mdanderson.org/depts/oncolog/articles/08/6-jun/6-08-2.html> Accessed: 28/07/2010.

MONZIR, A-L. Monzir-pal. [Online]. Available at: <http://www.monzir-pal.net/bioseparation/contents/highperformanceliquidchromatography.pdf> Accessed: 03/06/2011.

MORICE, P., SABOURIN, J.C., PAUTIER, P., GERBAULET, A., DUVILLARD, P. & CASTAIGNE D. 2000. Isolated para-aortic node involvement in stage Ib/II cervical carcinoma. *Eur J Gynaecol Oncol*. 21:123-125.

MSIBI, T.L. 2010. Personal communication from Dr. T.L. Msibi. Consultant. Department of Obstetrics and Gynaecology. University of Limpopo (Medunsa Campus). 15 March.

MURPHY, B.J., LADEROUTE, K.R., CHIN, R.J. & SUTHERLAND, R.M. 1994. Metallothionein IIA is up-regulation by hypoxia in human A 431 squamous carcinoma cells. *Cancer Res*. 54:5808-5810.

NECHAMKIN, H. 1968. *The Chemistry of the Elements*. New York: McGraw-Hill.

NGELANGEL, C., MÜNOZ, N., BOSCH, F.X., FESTIN, M.R., DEACON, J., JACOBS, M.V., SANTAMARIA, M., MEIJER, C.J.L.M. & WALBOOMERS, J.M.M. 1998. Causes of cervical cancer in the Phillipines: a case-control study. *F Natl Cancer Inst*. 90:43-49.

NTLOKWANA, A. 2011. Personal communication from Mr. A. Ntlokwana. Scientist. Technology Development. NTP Radioisotopes (Pty) Ltd. 1 December.

NUNN, A., LINDER, K. & STRAUSS, H.W. 1995. Nitroimidazoles and imaging hypoxia. *Eur J Nucl Med*. 22:265-280.

OVERGAARD, J. & HORSMAN, M.R. 1996. Modification of hypoxia-induced radioresistance in tumours by the use of oxygen and sensitizers. *Semin Radiat Oncol*. 6:10-21.

PARKIN, D.M., BRAY, F., FERLAY, J. & PISANI, P. 2002. Global cancer statistics, 2002. CA *Cancer J Clin*. 55:74-108.

PARKIN, D.M., WHELAN, S., FERLAY, J. & STORM, H. 2005. Cancer incidence in five continents, IARC Cancer Database No. 7, Lyon, 2005; Vols I-VIII.

PEREZ, C.A., CAMEL, H.M., ASKIN, F. & BREAUX, S. 1981. Endometrial extension of carcinoma of the cervix: a prognostic factor that may modify staging. *Cancer*. 48(1):170-180.

PETERS, W.A. 3RD., LUI, P.Y., BARRETT, R., STOCK, R.J., MONK, B.J., BEREK, J.S., SOUHAMI, L., GRIGSBY, P., GORDON, W. JR. & ALBERTS, D.S. 2000. Concurrent chemotherapy and pelvic radiation therapy compared with pelvic radiation alone as adjuvant therapy after radical surgery in high-risk early-stage cancer of the cervix. *J Clin Oncol*. 18:1606-1613.

PLANTE, M., RENAUD, M.C. & ROY, M. 2005. Radical vaginal trachelectomy: a fertility-preserving option for young women with early stage cervical cancer. *Gynecol Oncol*. 99(3 Suppl 1):S143-S146.

PUGH, C.W., O`ROURKE, J.F., NAGAO, M., GLEADLE, J.M. & RATCLIFFE, P.J. 1997. Activation of hypoxia-inducible factor-1: definition of regulatory domains within the alpha subunit. *J Biol Chem*. 272:11 205-11 214.

RASEY, J.S., KOH, W.-J., EVANS, M.L., PETERSON, L.M., LEWELLEN, T.K., GRAHAM, M.M. & KROHN, K.A. 1996. Quantifying regional hypoxia in human tumours with positron emission tomography of [18F] fluoromisonidazole: A pretherapy study of 37 patients. *Int J Radiat Oncol Biol Phys*. 25:417-428.

RASEY, J.S., NELSON, N.J., CHIN, L., EVANS, M.L. & GRUNBAUM, Z. 1990. Characteristics of the binding of labelled fluoromisonodazole in cells in vitro. *Radiat Res*. 122:301-308.

RASEY, J.S., KOH, W.J., EVANS, M.L., PETERSON, L.M., LEWELLEN, T.K., GRAHAM, M.M. & KROHN, K.A. 1996. Quantifying regional hypoxia in human tumours with positron emission tomography of [18F] fluoromisonidazole: A pretherapy study of 37 patients. *Int J Radiat Oncol Biol Phys*. 25:417-428.

REINHARDT, M.J., EHRITT-BRAUN, C., VOGELGESAND, D., IHLING, C., HORGELE, S., MIX, M., MOSEL, E. & KRAUSE, T.M. 2001. Metastatic lymph nodes in patients with cervical cancer: detection with MR imaging and FDG PET. *Radiology*. 218(3):776-82.

RICHARD, D.E., BERRA, E., POUYSSÉGUR, J. 1999. Angiogenesis: How a tumour adapts to hypoxia. *Biochem Biophys Res Commun*. 266:718-722.

ROSE, P.G., BUNDY B.N., WATKINS, E.B., THIGPEN, J.T., DEPPE, G., MAIMAN, M.A., CLARKE-PEARSON, D.L. & INSALACO, S. 1999a. Concurrent cisplatin-based radiotherapy and chemotherapy for locally advanced cervical cancer. *N Engl J Med*. 15:1144-1151.

ROSE, P.G., ADLER, L.P., RODRIGUEZ, M., FAULHABER, P.F., ABDUL-KARIM, F.W. & MIRALDI, F. 1999b. Positron emission tomography for evaluating para-aortic nodal metastasis in locally advanced cervical cancer before surgical staging: a surgicopathologic study. *J Clin Oncol*. 17(1):41-45.

SAHA, G.B. 2004. *Fundamentals of Nuclear Pharmacy.* 2nd ed. New York: Springer.

SEMENZA, G.L, NEJFELT, M.K., CHI, S.M. & ANTONARAKIS, S.E. 1991. Hypoxia-inducible nuclear factors bind to an enhancer element located 3` to the human erythropoietin gene. *Proc Natl Acad Sci USA*. 88:5680-5684.

SEMENZA, G.L. 1996. Transcriptional regulation by hypoxia-inducible factor 1; molecular mechanisms of oxygen homeostasis. *Trends Cardiovasc Med*. 6:151-157.

SINGH, A.K., GRIGSBY, P.W., DEHDASHTI, F., HERZOG, T.J. & SIEGEL, B.A. 2002. FDG-PET lymph node staging and survival of patients with FIGO stage IIIb cervical carcinoma. *Int. J. Radiation Oncology Biol Phys*. 56(2):489-493.

SLATTERY, M.L., ROBISON, L.M., SCHUMAN, K.L., FRENCH, T.K., ABBOTT, T.M., OVERALL, J.C. Jr. & GARDNER, J.W. 1989. Cigarette smoking and exposure to passive smoke are risk factors for cervical cancer. *FAMA*. 261:1593-98.

SONG, H., BOM, H., CHO, K.H., KIM, B.C., SEO, J., KIM, C., YANG, D.J. & KIM, E. 2003. Prognostication of recovery in patients with acute ischemic stroke through the use of brain SPECT with Technicium-99m labelled Metronidazole. *Am Heart Ass J*. 34:982-986.

STEPHMAN, F., BUNDY, B., DISAIA, P., P., KEYS, H.M., LARSON, J.E. & FOWLER, W.C. 1991. Carcinoma of the cervix treated with irradiation therapy. I. A multivariate analysis of prognostic variables in the Gynaecology Oncology Group. *Cancer*. 67:2776-2785.

SUBAK, L.L., HRICAK, H., POWELL, B., AZIZI, L. & STERN, J.L. 1995. Cervical carcinoma: computed tomography and magnetic resonance imaging for preoperative staging. *Obstet Gynecol*. 86:43-50.

SWEETMAN, S.C. (ed) 2002. *Martindale: The complete drug reference*. 33rd ed. London: Pharmaceutical Press.

TANAKA, H., YASUDA, Y., MUSHA, T., UEDA, K. & NAKAMURA, Y. 2005. Hypoxia correlates with angiogenesis in cervical cancers. *Int J Clin Oncol*. 10:35-39.

THEOBALD, T (ed). 2011. *Sampson's Textbook of Radiopharmacy*. 2nd ed. Cornwall: Pharmaceutical Press.

UNIVERSITY OF LIMPOPO (MEDUNSA CAMPUS). Nuclear Medicine Lectures. 2009. Radiopharmaceuticals in imaging studies. Unpublished.

UKRG HANDBOOK see UNITED KINGDOM RADIOPHARMACY GROUP

UNITED KINGDOM RADIOPHARMACY GROUP. Radiochemical Purity Systems of Radiopharmaceuticals (Page 9). [Online]. Available at: <http://www.ukrg.org.uk/rphandbook/radtlc.htm>. Accessed 23/11/2011.

VEENSTRA INSTRUMENTS. Quality Control System. [Online]. Available at: <http://www.veenstranet.com/docs/products/Nuclear%20Medicine/Quality%20Control%20Systems/Chromatography/Scanner%20Unit/Quality%20Control%20System.pdf>. Accessed 18/02/2011.

VERBRUGGEN, A.M., NOSCO, D.L., VAN NEROM, C.G., BORMANS, G.M., ADRIAENS, P.J. & DE ROO, M.J. 1992. Technetium-99m-$_{L,L}$-ethylenedicysteine: a renal imaging agent. I. Labelling and evaluation in animals. *J Nucl Med*. 33:551-557.

WAGGONER, S.E. 2003. Cervical cancer. *Lancet*. 361:2217-2225.

WECHALEKAR, K., SHARMA, G. & COOK, G. 2005. PET/CT in oncology- a major advance. *Clin Rad*. 60:1143-1155.

W.H.O. see WORLD HEALTH ORGANISATION

WORLD HEALTH ORGANISATION. International Pharmacopoeia. [Online]. 2008. W.H.O. Available at: <http://www.who.int/medicines/publications/pharmacopoeia/radsupplinfo.pdf>. Accessed: 08/07/2011.

YANG, D.J., ILGAN, S., HIGUCHI, T., ZARENEYRIZI, F., OH, C., LIU, C., KIM, E.E. & PODOLOFF, D.A. 1999. Noninvasive assessment of tumour hypoxia with ^{99m}Tc labelled metronidazole. *Pharm Res*. 16:5.

ZOLLE, I. 2007 *Technetium-99m Pharmaceuticals: Preparation and Quality Control in Nuclear Medicine.* Berlin: Springer.

APPENDICES

Appendix 1: UP Protocol Approval Certificate

Health Sciences, University of Pretoria comply with ICH-GCP guidelines and has US Federalwide Assurance. FWA 00002567, Approved dd 22 May 2002 and Expires 24 Jan 2009.
IRB 0000 2235 IORG0001762 Approved dd Jan 2006 and Expires 21 Nov 2008.

100 1908 - 2008

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

Fakulteit van Gesondheidswetenskappe Navorsingsetiekkomitee

Date: 10/04/2008

PROTOCOL NO.	**167/2007**
PROTOCOL TITLE	Imaging with Tc-99m Metronidazole – Prognostic Impact in Cervical Cancer
INVESTIGATOR	**Principle Investigator:** Prof. M. M. Sathekge
SUBINVESTIGATORS	Prof G Dreyer, Gynecological Oncology Unit, Obstetrics and Gynecology, Pretoria Academic Hospital; University of Pretoria Prof L Dreyer, Anatomical Pathology, Pretoria Academic Hospital, University of Pretoria Prof Z Lockhat, Radiology, Pretoria Academic Hospital, University of Pretoria Prof D Yang, Department of Nuclear Medicine, University of Texas M.D. Anderson Cancer Center, Houston, TX 2004, USA
DEPARTMENT	Nuclear Medicine, University of Pretoria, Pretoria Academic Hospital **Tel:** 012 354 1794 or 083 461 7653 **Fax:** 012 354 1219 **Email:** mike.sathekge@up.ac.za
STUDY DEGREE	No
SPONSOR	None.

This **Protocol** and **Informed Consent** and **all the attatchements** have been considered by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria on 26/03/2008 and found to be acceptable

*Advocate AG Nienaber	(female)BA(Hons) (Wits); LLB; LLM (UP); Dipl.Datametrics (UNISA)
*Prof V.O.L. Karusseit	MBChB; MFGP (SA); M.Med (Chir); FCS (SA): Surgeon
*Prof M Kruger	(female) MB.ChB.(Pret); Mmed.Paed.(Pret); PhDd. (Leuven)
Dr N K Likibi	MB.BCh.; Med.Adviser (Gauteng Dept.of Health)
*Snr Sr J. Phatoli	(female) BCur (Et.Al) Senior Nursing-Sister
*Dr L Schoeman	(female) Bpharm, BA Hons (Psy), PhD
*Dr R Sommers	(female) MBChB; M.Med (Int); MPhar.Med;
Mr Y Sikweyiya	MPH; Master Level Fellowship in Research Ethics; BSC (Health Promotions) Postgraduate Dip in Health Promotion
*Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path) Senior Specialist; Oral Pathology
*Dr A P van Der Walt	BChD, DGA (Pret) Director: Clinical Services of the Pretoria Academic Hospital
*Prof C W van Staden	MBChB; MMed (Psych); MD; FTCL; UPLM; Dept of Psychiatry

DR R SOMMERS; MBChB; M.Med (Int); MPhar.Med.)
SECRETARIAT of the Faculty of Health Sciences Research Ethics Committee - University of Pretoria

* Members attending the meeting.

HW Snyman Building(South) level 2-34■Private Bag X169 Pta. S.A. 0001■Tel:(012)354 1330.
Fax:0866515924■012-354 1367■E-Mail:manda@med.up.ac.za ■Web:http://www.healthethics-up.co.za

Appendix 2: MREC Protocol Approval certificate

UNIVERSITY OF LIMPOPO
Medunsa Campus

UNIVERSITY OF LIMPOPO

MEDUNSA RESEARCH & ETHICS COMMITTEE

CLEARANCE CERTIFICATE

P O Medunsa
Medunsa
0204
SOUTH AFRICA

Tel: 012 - 521 4000
Fax: 012 - 560 0086

MEETING: 09/2008

PROJECT NUMBER: MREC/H/223/2008: PG.

PROJECT :

Title:	Imaging with Tc-99 Metronidazole – Prognostic Impact in Cervical Cancer
Researcher:	Ms A Mdlophane
Supervisor:	Dr B Summers
Co Supervisor:	Prof M Sathekge (Nuclear Medicine) Pretoria Academic Hospital
Hospital Superintendents & Involved departmental Heads:	Prof G Dreyer, Gynecological Oncology Unit, Obseterics and Gynecology, Pretoria Academic Hospital, University of Pretoria Prof L Dreyer, Anatomical Pathology, Pretoria Academic Hospital, niversity of Pretoria Prof Z Lockhat, Radiology, Pretoria Academic hospital, University of Pretoria Prof D Yang, department of Nuclear Medicine, University of Texas M.D Anderson Cancer Center, Houston, TX 2004, USA
Department:	Pharmacy
School:	Health Care Sciences
Degree:	MSc (Med)

DECISION OF THE COMMITTEE:

MREC approved the project.

RESEARCH & PARTNERSHIP
2008 -11- 26
MEDUNSA CAMPUS

DATE: 26 November 2008

PROF GA OGUNBANJO
CHAIRPERSON MREC

Note:
i) **Should any departure be contemplated from the research procedure as approved, the researcher(s) must re-submit the protocol to the committee.**
ii) **The budget for the research will be considered separately from the protocol.**
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.

African Excellence - Global Leadership

Appendix 3: Case Record Form

CRF: Imaging with Tc-99m Metronidazole – Prognostic Impact in Cervical Cancer
TRIAL NUMBER:167/2007

Case Record Form

Patient Name: _____Age:_____Gender: M/F_____

Weight (Kg):_____Height (cm):_____

BMI:_____ Waist/Hip Ratio:_____

Diagnosis of Cervical cancer:

Cervical biopsy Date:	
Tumour size	
LVI	
Grade	

Blood investigations:

HIV status	
CD 4 count	
Full blood count	
Kidney function test	
Liver function test (including albumin)	

Ultrasonography:

Kidneys	
Bladder	

Imaging:

Findings on CXR: _____

Histological evaluation:

Routine staging and tumour characteristics	
Immunohistochemical measures of hypoxia [vascular endothelial growth factor (VEGF)]	
Histological measures of hypoxia using tumor vascularity [microvessel density(MVD)]	

^{99m}Tc-EC-MN imaging

Findings on SPECT/CT: _____

Location	Severity	T/B at 30 min	T/B at 2 hrs	Retention Index

Details of treatment: _____

_____

Final diagnosis at discharge: _____

Date of follow up :_____

Appendix 4: Patient Information and Informed Consent Form

PATIENT INFORMATION AND INFORMED CONSENT DOCUMENT

TRIAL NUMBER:……..

TRIAL TITLE:

Imaging with Tc-99m Metronidazole – Prognostic Impact in Cervical Cancer

INTRODUCTION

You are invited to take part in a study involving 10 patients. This document will help you to decide whether you would like to participate. Before you agree to take part in this study you should fully understand what it is all about. If you have any questions that are not fully explained in this document, do not hesitate to ask the study doctor. You should not agree to take part unless you are completely happy about all the procedures involved.

WHAT IS THE PURPOSE OF THIS TRIAL?

You have been diagnosed with cervical cancer. In addition to the routine work up for cervical cancer, an additional SPECT images will be obtained. The SPECT scanner is routinely used in the nuclear medicine department with various cancers for detection of metastases, recurrence of cancer, and renal function in cancer patients. The scanner also gives indications whether the heart is working properly after chemotherapy. In this case the scanner might help us to diagnose whether the cervical cancer has deficiency of oxygen or not, the level of oxygen supply may help predict the response to treatment. The procedure goes as follows: An intravenous (IV) line will be inserted in your arm. This is similar to a pinprick when having blood drawn.

The radiopharmaceutical (^{99m}Tc-EC-MN) is then injected through the IV. A radiopharmaceutical is a very small amount of pharmaceutical that is tagged with a tiny amount of radioactive material. The amount of radioactivity you will receive from a ^{99m}Tc-EC-MN study is equivalent to the amount you would receive from other conventional scans that have been used for decades. There is no published/reported data that any side effects in humans have been directly related to radiation exposure subjects have received from ^{99m}Tc-EC-MN SPECT. In our day to day activities, we are exposed to various types of naturally and artificially occurring radiation, for instance flying as long as 10 hours in an aeroplane will expose the body to 0.1 mSv.

You will wait approximately 30 minutes until the ^{99m}Tc-EC-MN circulates in your body. For approximately 20-30 minutes, while you are lying down, we will take pictures of your body and pelvis with a SPECT camera. The functionality of the SPECT camera procedure has been shown to be very safe in millions of patients and is used daily in nuclear medicine departments. Your body is able to quickly eliminate the radioactive materials used.

WHAT IS THE DURATION OF THIS TRIAL?

We will enrol patienst to this study, which will probably be completed in 12 months.

SPECT scan after diagnosis but before the beginning of the treatment. The whole procedure will take about 3 hours. The scanning itself will take about 60 minutes (30 minutes 1 hour after injection, 30 minutes 2 hours after injection).

HAS THE TRIAL RECEIVED ETHICAL APPROVAL?

The protocol of this clinical trial will be submitted to the ethical committee of the University of Pretoria for written approval. The study has been structured in accordance with the Faculty of Health Sciences Research Ethics and Declaration of Helsinki (last updated in 2004), which deal with the recommendations guiding doctors in biomedical research involving human participants. Copies of these documents can be obtained from a study doctor should you wish to review it.

WHAT ARE YOUR RIGHTS AS A PARTICIPANT IN THIS TRIAL?

Your participation in this trial is entirely voluntary and you can refuse to participate or stop at any time without stating any reason. Your withdrawal will not affect your access to other medical care. The study doctor retains the right to withdraw you from the study if it is considered to be in your best interest.

If it is detected that you did not give an accurate history or did not follow the guidelines of the trial and the regulations of the trial facility, you may be withdrawn from the trial at any time.

MAY ANY OF THESE TRIAL PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE ?

Installation of the intravenous line might be uncomfortable and even slightly painful. It might also result in bruise formation and infections. Administration of the radiopharmaceutical (^{99m}Tc-EC-MN) in the vein might be uncomfortable and even slightly painful. It might also result in bruise formation and infections.

All these procedures will however be done only by well-experienced health personnel, under hygenic conditions, and in your best interest.

WHAT ARE THE RISKS INVOLVED IN THIS TRIAL?

There will be no other risks involved in this trial apart from the complications mentioned in the previous paragraph.

FINANCIAL ARRANGEMENTS

The additional ^{99m}Tc-EC-MN scans will be financed by the Department of Nuclear Medicine University of Pretoria.
The additional immunohistochemistry staining exams done at the NHLS will be negotiated and paid from the existing research fund.

COSTS

You will not be paid to participate in this trial. You will also not be asked to pay anything more than the usual fees required for admission to hospital.

SOURCE OF ADDITIONAL INFORMATION

For the duration of the trial, you will be under the care of Prof Mike Sathekge and Prof Greta Dreyer. If at any time between your visits you feel that any of your symptoms are causing you problems, or you have any questions, please do not hesitate to contact them. The telephone number through which you can reach them is 012 354 1794 or 012 354 2366 or casualty at 012 354 2384. You must notify a study doctor immediately if you experience complications and/or injuries resulting from the trial.

CONFIDENTIALITY

All information obtained during the course of this trial is strictly confidential. Data that may be reported in scientific journals will not include any information that identifies you as a patient in this trial.

In connection with this trial, it might be important for domestic and foreign regulatory health authorities, the Faculty of Health Sciences Research and Ethics Committee of University of Pretoria , the Medicines Control Council, as well as your personal doctor, to be able to review your medical records pertaining to this trial. Therefore, by signing this document, you authorise your study doctor to release your medical records to study coordinators involved in this study, regulatory health authorities, the Medicines Control Council. You must understand that these records will be utilised by them only to carry out their obligations related to this clinical trial.

You will be informed of any finding of importance to your health or continued participation in this trial.

INFORMED CONSENT

I have read, or had read to me in a language that I understand, the above information before signing this consent form. The content and meaning of this information have been explained to me. I have been given opportunity to ask questions and I am satisfied that they have been answered satisfactorily. I understand that if I do not participate it will not alter my management in any way.

I hereby volunteer to take part in this study.

_____ _____.
Patient / Guardian (signature) Date

_____ _____.
Person obtaining informed consent (signature) Date

_____ _____.
Witness (signature) Date

VERBAL PATIENT INFORMED CONSENT
(applicable when patients cannot read or write)

I, the undersigned, Dr ……, have read and have explained fully to the patient, named ……and/or his/her relative, the patient information leaflet, which has indicated the nature and purpose of the trial in which I have asked the patient to participate. The explanation I have given has mentioned both the possible risks and benefits of the trial and the alternative treatments available for his/her illness. The patient indicated that he/she understands that he/she will be free to withdraw from the trial at any time for any reason and without jeopardizing his/her subsequent injury attributable to the drug(s) used in the clinical trial, to which he/she agrees.

I hereby certify that the patient has agreed to participate in this trial.

Patient's Name _____
(Please print)

Investigator's Name _____
(Please print)

Investigator's Signature _____ Date _____

Witness's Name _____
(Please print)

Witness's Signature _____ Date _____