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Quantitative Evaluation of the Carbon Isotopic Labelled Urea Breath Test for the Presence of Helicobacter pylori

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Quantitative Evaluation of the Carbon Isotopic Labelled Urea Breath Test for the Presence of *Helicobacter pylori*

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A research report presented to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg in partial fulfilment of the requirements for the degree of Master of Science in Medicine.

Declaration

I declare that this research report is my own unaided work. It has not been submitted before for any degree or examination at any other University.

_____

Day of 2005

Abstract

The ^{14}C and ^{13}C labelled urea breath tests (UBT) for detecting *Helicobacter pylori* infection are well established but scope for improvement exists in both to reduce some of their shortcomings.

For this study, the ^{14}C UBT investigation focussed on reducing the quantity of radioactive tracer that is administered to the subject undergoing this test, with the aim of lowering the radiation dose to the patient, reducing the impact to the environment and exempting the test from radioactive materials licensing. Wider acceptance, availability, affordability to lower socio-economic groups and third party medical treatment payers and using readily available equipment were factors considered when developing the method.

The principle of the method developed is to collect larger volume breath sample, quantitatively absorbing a defined volume of extracted breath CO_2 in an efficient CO_2 trapping agent using a specifically designed apparatus and measuring the activity with a low background β-spectrometer.

A reduction in the quantity of ^{14}C labelled urea administered to the patient was achieved. The method also reduced the counting error margin at a lower detection limit, improving discrimination between *H. pylori* positive and negative patients.

The ^{13}C UBT is a non-radioactive test however, it is substantially more expensive. The ^{13}C UBT investigation aimed to determine whether commercially available un-enriched urea could be used thus reducing the cost of the ^{13}C UBT.

A simple protocol with Isotope Ratio Mass Spectrometry (IRMS) for the measurement was used as opposed to the well-established ^{13}C UBT protocol. The principle of the ^{13}C UBT investigation was to detect the change of the breath $\delta^{13}C$ ($^{13}C/^{12}C$) ratio after the administration of un-enriched urea with a $\delta^{13}C$ different to the exhaled breath. Theoretical calculations showed that an administered dose of 500mg un-enriched urea with at least a 10‰ $\delta^{13}C$ difference may be detectable using IRMS.

In vitro investigations confirmed that levels of 0.01 to 0.001‰ $\delta^{13}C$ were detectable by IRMS. A change in the $\delta^{13}C$ of a standard breath CO_2 was confirmed for a range between 0.14 to 50% $^{v}/_{v}$ mixed CO_2 samples, i.e. the projected range for *in-vivo* investigation. Results from the *in-vivo* investigation however were not able to distinguish positive from negative *H. pylori* patients. The use of the 1000mg dose of urea appears to have caused saturation of the enzyme. It was concluded that some enrichment of the ^{13}C is necessary or less urea be used.

I dedicate this report to and share the report with my wife Susan, my daughter Adriana, and my sons, Alwyn, Ayrton and Adriaan for their love, support, encouragement, understanding and patience.

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Nomenclature

AMS - Accelerator Mass Spectrometry

ALARA - As Low As Reasonably Achievable

Bq - Becquerel

CPM - Counts Per Minute

DPM - Disintegrations Per Minute

FDA - Food and Drug Administration, USA

GERD - Gastro-Oesophageal Reflux Disease

GIT – Gastro Intestinal Tract

IAEA - International Atomic Energy Agency

IARC - International Agency for Cancer Research

ICRP - International Commission for Radiation Protection

IRMS - Isotope Ratio Mass Spectrometry

LARA - Laser Assisted Ratio Analysis

MALT - Mucosal-Associated-Lymphoid-Type lymphoma

PDB – Pee Dee Belemnite (Reference standard for $\delta^{13}C$)

PPI – Proton Pump Inhibitor

PUD - Peptic Ulcer Disease

SIS – Spectral index of sample

Sv - Sievert

tSIE – Transformed spectral index of external standard

UBT - Urea Breath Test

CHAPTER 1

1.1 Introduction

1.1.1 Historical background

Helicobacter pylori was originally described by Marshall and Warren in 1983 [1] and named *Campylobacter pyloridis* because of its structural similarities to the other *Campylobacter* species [2]. It was renamed to *C. pylori,* and *Helicobacter pylori* in 1989 [3] as specific morphologic, structural and genetic features indicated that it should be placed in a new genus.

Before the description of this bacterium, spicy food, acid, stress, and lifestyle were considered the major causes of ulcers, although descriptions of bacterial associated damage to the gastric mucosa of animals and humans date back approximately 100 years [4]. The majority of these patients were treated with drugs including, histamine receptor inhibitors (H2 receptors), and more recently, proton pump inhibitors (PPi) [5]. Surgical interventions like a "selective vagotomy" were often also performed. These medications and interventions only relieved the ulcer-related symptoms and may have healed gastric mucosal inflammation and the ulcer, but did not treat the *H. pylori* infection. When acid suppression treatment was removed, the majority of ulcers recurred [6].

The isolation and description of *H. pylori* and its association with inflammatory gastric disease is undoubtedly one of the major medical breakthroughs in the last 20 years. Since then the management of upper gastro-intestinal tract (GIT) disease has changed radically as peptic ulcer disease (PUD) is treated primarily as an infectious disease [(7)].

H. pylori is classified as a group I (definite) carcinogen in humans by the World Health Organization's International Agency for Cancer Research (IARC) [(8)], therefore making it an important factor to consider in the treatment and prognosis of ulcerative gastritis, peptic ulcer disease, gastric cancer and mucosa-associated lymphoid-tissue (MALT) lymphoma [(9)].

Another spiral urease producing bacterium *H. helimannii*, found in dogs, cats, pigs, and non-human primates can infect humans but prevalence is only about 0.5% [(10)]. This infection causes only mild gastritis in most cases, although association with MALT lymphoma has been reported [(11)].

1.2 Background

1.2.1 Description of *H. pylori*

Helicobacter pylori as shown in Figure 1 and 2, is a spiral-shaped, gram-negative, curved rod bacterium approximately 0.5 x 3.0 micrometers in size. This oxidase, catalase and urease positive organism has 4-6 sheathed flagella attached to one pole that allow motility [12].



Figure 1: Scanning electron microscope photo of *H. pylori* [12]

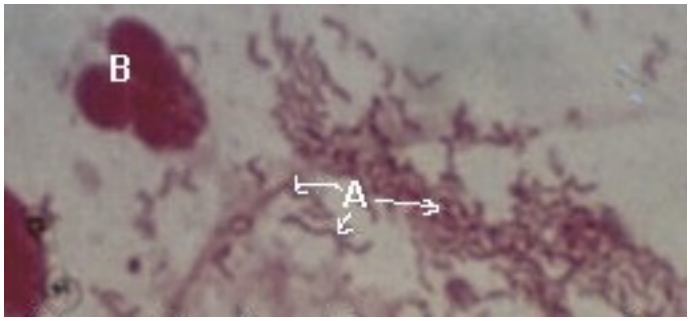

Figure 2: Gram stain of a gastric mucus smear from a patient with duodenal ulcer. Many curved bacilli (A) are seen together with a polymorphonuclear leukocyte engulfing some bacteria (B), (1000X magnification) [12]

1.2.2 Epidemiology

Infection with *H. pylori* occurs worldwide, with the prevalence varying between countries and among population groups within the same country. Strong associations exist between socio-economic conditions and prevalence of *H. pylori* infection where about 80 percent of middle-aged adults in developing countries are infected, compared to 20 to 50% in industrialized countries [13].

The infection is acquired in early childhood [14] by oral ingestion of the bacterium and transmission is mainly direct from person to person within families via saliva, vomitus, faeces or contaminated foods, the latter particularly from street vendors [13]. In developing countries, unpurified water may be an important additional source of infection where *H. pylori* is present in more than 75 percent of tested surface drinking water samples [15].

Smoking, alcohol, and gender do not appear to influence the prevalence of *H. pylori* but studies of twins in Sweden have suggested that there is a considerable genetic component in the susceptibility to infection [16].

However, a better understanding of the entire spectrum of disease caused by *H. pylori* is still required, particularly in identifying popula-

tions and groups that might benefit from eradication treatment before the infection progresses to overt disease. More studies are also needed among children, because early acquisition of the infection appears to determine the subsequent development of complications, including gastric cancer and gastric ulcer [13, 17].

1.2.3 Pathogenesis

H. pylori is able to colonise the gut by using its own motility within the viscous gastric mucous layer, evading both gastric motility and peristalsis. The bacterium usually colonises the antrum [18] region of the stomach first because of the moderate acid levels in that region [12]. The organism seems to selectively overlay and bind to gastric-type epithelial cells in the stomach or metaplastic duodenal cells, avoiding the absorptive-type duodenal cells [12].

The bacterium *H. pylori* produces urease [19] which is the most active of known ureases with the reported Michaelis constant of ± 0.3 mM [18]. The ammonia generated around the bacteria creates a less acidic microenvironment that is an essential condition for the first step of infection [19]. The enzyme urease that converts urea, even at very low concentrations to ammonia and carbon dioxide, appears both on the surface and in the cytoplasm of the bacterium [18].

The majority of *H. pylori* strains produce cytotoxins (e.g. CagA and VacA) but, is complicated by the diversity and variability in the vacA gene. Atheron et al [20] suggests the association of vacA gene with a more severe disease in western countries however, this association has not been found in Asia. Detection of these cytotoxin specific *H. pylori* strains would allow for an aggressive therapeutic approach [13].

H. pylori induce vigorous systemic and mucosal immune responses in the host [21] through secretion of bacterial proteases, phospholipases and the cytotoxins. These responses are however ineffective against the bacterium in the mucus lining and contribute to the inflammatory response [21] with the generation and deposition of other destructive compounds (superoxide radicals) when the polymorphonuclear leukocytes die [22]. Further injury by hydrogen ions occurs when mucosal cell membranes are damaged. *H. pylori* itself may therefore not directly cause peptic ulcer, which may be caused by the inflammatory response to the bacteria.

Epidemiological studies have linked *H. pylori* to an increased risk for the development of gastric cancer [17, 23]. It is classified as a definitive carcinogen by the IARC [8]. However, details of the pathogenic mechanism remain unclear.

1.2.4 Clinical Outcome

The clinical course of the *H. pylori* infection is variable, influenced by microbial and host factors. The infection always causes gastritis, often asymptomatic, and identified only microscopically [24]. The role of *H. pylori* in dyspepsia is still uncertain showing no benefit to treatment [25] however, *H. pylori* is responsible for nearly 100% of duodenal ulcers and 60% to 80% of gastric ulcers. Its association with gastric adenocarcinomas and MALT lymphomas suggests an estimated six times increased risk of these neoplasms [17].

H. pylori positive persons who have developed peptic ulcer disease may have acquired the infection up to 10 years earlier [23].

1.2.5 Diagnostic tests for *H. pylori*

Invasive or non-invasive methods confirm the *H. pylori* infection status of a person [26, 27, 28, 29] and diagnostic testing for *H. pylori* infection is indicated in patients suffering from peptic ulcer disease, gastric ulcer disease, gastric carcinoma and MALT lymphoma [26].

1.2.6 Invasive technique

The invasive techniques require biopsy specimens of the stomach lining from the subjects. These specimens are usually obtained by an upper gastro intestinal endoscopy procedure, with its associated risks and discomfort to the patient [27, 29]. It is a discrete sampling technique that requires specialized skills and needs to be carried out in a hospital setting.

Patients presenting with anaemia, gastro-intestinal bleeding, weight loss and patients older than 50 years of age are indications for an invasive diagnostic procedure such as endoscopy to sample directly for *H. pylori*, as it provides the added diagnostic benefit for evaluating associated pathology. The *H. pylori* test of choice in these patients using these procedures is the rapid urease test [28].

1.2.6.a Culture

H. pylori can be cultured from a biopsy specimen or the homogenate with various methods for at least seven days at 37°C on specialised agar plates [28, 29].

Such cultures, by definition being 100% specific, are regarded as the "gold standard" for detecting *H. pylori*. The discrete sampling tech-

nique however limits diagnostic sensitivity to 60 - 90% [29]. Furthermore, the comparative cost for each test is high with results only being available at least a week after testing [29].

1.2.6.b Histology

Histological examination of a haematoxylin/eosin or Giemsa stained biopsy samples permits identification of the *H. pylori* and at the same time allows evaluation of tissue damage and possible dysplastic changes [29]. Comparative studies between the different staining techniques have not clearly shown one to have an advantage over the other.

Histology has a lower sensitivity (80 - 95%) than culture but specificity may range up to 100% depending on the experience of the pathologist examining duplicate samples. Confirmation of the results also requires a waiting period of a few days [18, 27, 29].

1.2.6.c Rapid Urease test

H. pylori produce large amounts of the urease enzyme which catalyses the hydrolysis of urea to CO_2 and NH_3. This feature together with the otherwise low activity of urease in a healthy human gastro-intestinal tract is the basis of the rapid urease tests of biopsy specimens [27, 29].

The biopsy specimen is placed in a test solution containing urea, a pH colour reagent and a bacteriostatic agent. A change in colour resulting from the hydrolysis of urea to alkaline ammonia indicates a positive test. This normally occurs from one to twenty-four hours depending on the commercial test used [27, 29].

The test itself is relatively inexpensive but obtaining the biopsy specimen still requires an invasive endoscopy procedure. The technique is 90 - 95% sensitive with similar specificity.

1.2.6.d Molecular biology

Polymerase chain reaction (PCR) is generally useful only in the research environment for sub-typing *H. pylori*. Possible clinical use may be to distinguish recrudescence from re-infection in post-eradication treatment relapse [18].

1.2.7 Non-invasive testing

1.2.7.a Serology

Serologic tests are performed on blood, serum or saliva samples to detect antibodies specific to the *H. pylori* bacterium antigens. These tests are strain specific and in comparison to the invasive techniques, have sensitivity and specificity of about 95% [27, 29].

Serological evaluation methods of blood samples by enzyme-linked immunoassays (ELISA), Western Blot or Recombinant ImmunoBlot Assay (RIBA) are simple, quick and accurate with minimal discomfort and risk to the subject [18]. The costs of antibody tests (bedside or laboratory) are much lower than any of the other methods [30]. Results could become available within a few minutes from a blood sample obtained from a small finger prick [27, 29].

A major disadvantage of all serology tests is that a positive test result does not distinguish between current and past *H. pylori* infection because the IgG antibodies against *H. pylori* remain at raised levels in the blood for a long period (6-12 months and longer) after treatment [31, 32]. Therefore, it cannot be utilised as an indicator of eradication efficacy. These tests however, will not give false negative results in

patients who have taken a combination of antibiotics, bismuth compounds or proton pump inhibitors in the recent past [4, 32].

1.2.7.b *H. pylori* stool antigen test

H. pylori antigen may be directly detected in stool specimens, using for example the sandwich enzyme-immunoassay method [33], to provide the diagnosis of an active infection. The stool test therefore allows for both infection diagnosis and more importantly eradication treatment efficacy monitoring [34]. The European *Helicobacter* Study Group proposed this test as an alternative to the urea breath test in post treatment diagnosis [35]. Sensitivity and specificity of this test range between 85% and 95% and the cost of the test is low [36].

The *H. pylori* stool test can be conveniently performed in young children making it the test of choice for this group of patients [13]. The HpSA test (Meridian Diagnostics, Cincinnati) has achieved recent FDA approval.

1.2.7.c Breath testing

Urea breath testing exploits the urease produced by *H. pylori*, which catalysis the hydrolysis of orally administered urea into CO_2 and NH_3. These products diffuse into the blood and are exhaled, where their concentrations can be determined [37, 38, 39].

The UBTs are functional tests of the urease activity in the stomach, sampling non-discretely almost the entire stomach allowing for the diagnosis of an active infection and therefore permits post-treatment eradication monitoring [34, 35, 40]. Sensitivity and specificity of the UBTs are 95% and 96% respectively [34]. As with all the other tests except serology, the UBTs are unreliable in patients who have been taking antibiotics, bismuth, or proton pump inhibitors [41] during the weeks prior to the test.

A disadvantage of the ^{14}C UBT is the minor radiation exposure to the subject from the radioactive ^{14}C isotope making the non-radioactive ^{13}C UBT, despite being significantly more expensive, the preferred method when evaluating children or pregnant women [26, 28].

The FDA has approved the ^{14}C PY test® (Tri-Med Specialities, Charlottesville, VA) [42] and the ^{13}C BreathTek® (Meretek Diagnostics Inc.) breath tests [43]. The UBTs are discussed further in CHAPTER 2.

1.2.8 Summary of Diagnostic Tests

Table 1 summarises the diagnostic tests in routine clinical use with cost factor included, but the choice and application of a particular test should be based on sound clinical principles also taking the specific indications of each test into account.

Table 1 Summary of *H. pylori* diagnostic tests

Method	Culture	Histology	Rapid Urease	^{13}C UBT	^{14}C UBT	Serology	Stool test
Sensitivity %	60-95	90–95	80–95	95–100	95–100	85–95	90–95
Specificity %	100	95-98	95-100	88–98	88–98	75–90	90–95
Invasive	Yes	Yes	Yes	No	No	No	No
Cost	High	High	Moderate	Moderate	Low	Lowest	Low
Discrete sampling	Yes	Yes	Yes	No	No	No	No
Affected by recent ulcer treatment	Yes	Yes	Yes	Yes	Yes	No	Yes
Major advantage	Specificity	Evaluation of tissue damage and metaplasia	Results within an hour Evaluation of tissue damage	Not radioactive	Equipment readily available	Quick	Indicate active infection
Major disadvantage	Results only week later		CLO test needs 24 hours	Isotope expensive	Radioactive exposure	Does not reflect active infection	Laboratory preparation and special transport

1.2.9 Treatment of *H. pylori* infection

1.2.9.a Treatment indication

Eradication of *H. pylori* is the primary goal of treatment in patients with peptic ulcer disease, gastric ulcer disease, atrophic gastritis, MALT lymphomas, metaplastic gastric changes, and in patients and their relatives with gastric carcinoma [26, 28, 35]. Regression of low-grade MALT lymphoma has been shown to occur with *H. pylori* eradication [9, 11, 44].

There is insufficient evidence to justify the widespread use of eradication treatment in patients with dyspepsia or other symptomatic or asymptomatic conditions [25, 28]. Treatment may however be offered to any person testing positive for *H. pylori* infection, but potential risks such as antibiotic associated pseudo-membranous colitis from *Clostridium difficile* infection [25, 45] should be considered.

1.2.9.b Treatment regimen

Eradication of *H. pylori* is important in the identified risk groups where treatment is indicated, and for the infection to be treated optimally. Eradication is difficult because of the poor transfer into and the short residence time of the antibiotic in the stomach. *H. pylori* is also capable of developing resistance to the commonly used antibiotics. Therefore, a double or triple therapy with two or more antibiotics in combination with a proton pump inhibitor (PPI) and/or bismuth containing compounds is normally prescribed with over 90% eradication efficacy for *H. pylori* infection in PUD. The FDA and the European *Helicobacter* Study Group (Maastricht 2000 consensus) recommended therapy with 3-4 drugs [35]. One recommended treatment regimen is PPI (lansoprazole 30 mg) + amoxycillin 1000 mg + clarithromycin 500 mg per day, with each drug given in two divided doses for at least seven days [35].

Side effects including gastrointestinal disturbances, nausea from clarithromycin and bad taste from bismuth result in poor or reduced patient compliance with reduced eradication rates.

1.2.9.c Treatment failure

Antibiotic resistance of *H. pylori* and poor patient compliance are responsible for most treatment failures. About half of the strains show resistance to metronidazole. Although rare, the resistance to amoxycillin and clarithromycin is increasing [46].

A number of different therapeutic regimens are currently being compared to improve efficacy.

1.2.10 Prognosis and clinical outcome

1.2.10.a *H. pylori* eradication and ulcer relapse rates

Peptic ulcers relapse rate is over 80% within one year amongst patients treated only with histamine antagonists and/or proton pump inhibitors. However, eradication of *H. pylori* with antibiotic therapy cures the ulcer and reduces the one-year relapse rate to fewer than 10% [47] with a reported relapse of 3.7% per patient year in South Africa [48].

1.2.10.b Recurrence of infection

Recurrences of *H. pylori* infection after apparently successful eradication result from either recrudescence or re-infection. Recrudescence is the re-appearance of a *H. pylori* strain identical to the pre-treatment strain after a negative initial post-treatment assessment whereas re-infection has a genetically different post-treatment *H. pylori* strain from that before eradication treatment [49].

Evidence shows that recrudescence accounts for the vast majority of *H. pylori* infection recurrences [49] with the highest rate immediately after eradication therapy, diminishing with follow-up time to [50] less than 1% per year after the first year [51].

CHAPTER 2

2.1 Urea Breath Tests

2.1.1 Introduction

The three major isotopes of carbon are ^{12}C, ^{13}C and ^{14}C. ^{12}C and ^{13}C are stable carbon atoms, with natural abundance of approximately 98.9% and 1.1% (11‰) respectively [52]. The radioactive ^{14}C isotope occurs naturally at 10^{-10}% [53].

2.1.1.a ^{13}C background

^{13}C is a non-radioactive isotope. Its abundance relative to the major stable carbon isotope ^{12}C is expressed as the "delta value" ($\delta^{13}C$) on a per-mille (‰) scale. $\delta^{13}C$ is defined by $((R_{sample}-R_{std})/R_{std})\times 1000$, where $R=^{13}C/^{12}C$ for both sample and an accurately known reference standard [52, 54]. The international reference standard for ^{13}C is the PDB standard from belemnite of the Pee Dee Formation in South Carolina, with $\delta^{13}C$ value of 11.237‰ [53]. Fractionation causes variation in the ^{13}C natural occurrence in the range between 0 and -50‰ with marine carbonate rocks having $\delta^{13}C$ values of 0 to -2‰, C4 plants between 0 and -15‰ and C3 plants ranging between -20 to -30‰ allowing identification up to ppm accuracy for different sources of ^{13}C with Isotope

Ratio Mass Spectrometry (IRMS). This fingerprinting with $\delta^{13}C$ is carried out in such varied disciplines as geology and archaeology.

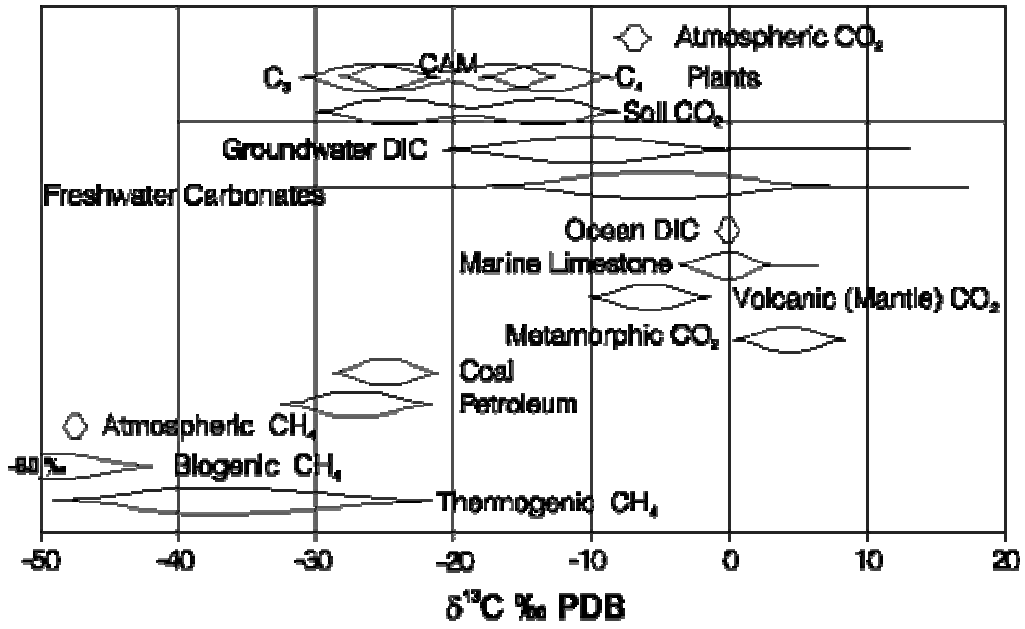

Figure 3: $\delta^{13}C$ values of naturally occurring carbon [52]

2.1.1.b Measuring ^{13}C

Techniques used to measure the ^{13}C proportion include IRMS (precision 0.01 to 0.005‰) and laser-based detection methods such as diode laser-based direct absorption spectroscopy. The latter having a lower accuracy of 0.8‰ [55].

2.1.1.c ^{14}C Background

Natural production of the radioactive isotope ^{14}C occurs in the atmosphere by cosmic ray neutron interactions with ^{14}N nuclei forming ^{14}C,

which is then oxidized to $^{14}CO_2$. ^{14}C decays by internal conversion emitting only β radiation with maximum and mean energy of 156keV and 49keV respectively. The radioactive half-life of the ^{14}C is 5730 years. This ^{14}C production exists in equilibrium with its radioactive decay giving the atmospheric pool an abundance $^{14}C/^{12}C$ ratio of 10^{-12}, a value that has been assigned the value of 1.00 and defined as 100% modern carbon (PMC). Nuclear testing in the 1950's and 1960 has increased this ratio to produce the "^{14}C bomb peak" of more than 100% but this level is returning to the pre-1950's level. ^{14}C activity of normal living matter decays at ±15 disintegrations per minute per gram of carbon [53].

2.1.1.d Measuring ^{14}C

The low energy β radiation is difficult to detect externally to the source, as the surrounding media easily absorb the electrons. Its range in air is of the order of a few millimetres. Special techniques are therefore required for its detection. These include liquid scintillation spectrometry [56], accelerator mass spectrometry (AMS) [57], and gas ionisation amplification.

Liquid scintillation spectrometry detects the photons generated from the interaction of the β radiation with the fluorescent matrix in which the sample is mixed. It is the more universally applied method for ^{14}C measurement because of readily available equipment, simple and fast

sample preparation, much cheaper than AMS, and is less labour intensive than gas amplification.

The ^{14}C abundance is then expressed as counts per minute (cpm), disintegrations per minute (dpm) or disintegrations per second (Bq). This can be related to reference levels like percent modern carbon (PMC) and/or age for natural occurring samples, like dating ancient artefacts up to 50,000 years [56].

2.1.2 Principle of UBT

With the UBT, a fasting patient orally ingests a small amount of ^{14}C or ^{13}C labelled urea, which is hydrolysed by *H. pylori* urease in the stomach to NH_3 and labelled CO_2. This CO_2 diffuses into the blood, and its concentration is measured in exhaled breath [37, 38].

A positive result identifying a *H. pylori* infection for ^{13}C- or ^{14}C UBT is indicated as a significant change or increase in the exhaled breath $\delta^{13}C$ values or ^{14}C concentration respectively between pre-ingestion (baseline) and post-ingestion (test) samples [37, 38].

2.1.3 Advantages and disadvantages of UBT [13, 29, 36, 38]

The UBT has many advantages over comparable tests by:

- being non-invasive,
- being well tolerated by the subjects,
- being a functional test for *H. pylori* urease reflecting the present status of infection,
- being cost effective and relatively inexpensive,
- being highly sensitive and specific (>90%),
- demanding minimal time and effort from the patients,
- not requiring highly trained personnel to perform,
- allowing the possibility of the tests being carried out at private practice level,
- providing results within hours.

A disadvantage of the ^{14}C UBT is that the radioactive urea exposes the subject to a small amount of ionising radiation [58].

The ^{13}C labelled urea and the analysis equipment is considerably more expensive than the ^{14}C labelled urea and its measuring equipment. IRMS spectrometers are also not suitable for field deployment due to their large physical size resulting in the ^{13}C UBT being less frequently used and not as generally available as the liquid scintillation spectrometers used for ^{14}C analysis [27]. However, techniques using smaller

and less expensive equipment such as LARA (laser assisted ratio analysis) are becoming available, leading to an increase in ^{13}C UBT usage frequency [55].

2.1.4 Indications for UBT

The primary indication for UBT is the monitoring of treatment efficacy i.e. verification of *H. pylori* infection eradication. A 4-8 week post treatment delay is recommended before a UBT is done to reduce false negative results [35, 59, 60].

UBT is also used to confirm *H. pylori* infection in patients with bleeding and recurrent ulcerative gastritis and PUD. This confirmation is necessary as *H. pylori* is not the only cause of gastro-intestinal ulcers and bleeding, and this would optimise treatment [25].

2.1.5 UBT accuracy

UBTs have high accuracy and reproducibility as it not subject to discrete sampling errors that biopsy-based methods may have.

Several reports have suggested that the breath tests should become the "gold standard" for *H. pylori* diagnosis [35, 61], and used in preference to culture or rapid urease tests.

2.1.5.a Sensitivity (percentage true positive)

As noted, the average sensitivity of the UBTs is about 95% making false positive results uncommon but may occur if there is intra-oral hydrolysis of urea by bacteria. However, this will usually produce an early peak in labelled CO_2 excretion and should not interfere with the test for *H. pylori*. Rarely do other urease-producing bacteria such as *H. helimannii* infect the human stomach; the prevalence is low (±0.5% in the population [9]).

2.1.5.b Specificity (percentage true negatives)

The average specificity of the UBT is also about 95% and can be further increased by strict adherence to the protocol. False-negative tests may occur if the patient took therapeutic medication, such as anti-secretory drugs like bismuth, PPI or antibiotics within the two weeks leading up to the test, whether given for the treatment of *H. pylori* infection or another unrelated reason [35, 41].

2.1.6 Dosage

2.1.6.a ^{13}C UBT

The administered urea dose for regular ^{13}C UBT is between 75 and 100mg. For reasons of cost, efficiency and practicability, a number of investigations reported successful reduction of the urea dose to 50 mg while maintaining excellent diagnostic accuracy [62].

2.1.6.b ^{14}C UBT

At present, many countries including South Africa and Chile are still using the ^{14}C breath test with an activity concentration of 370 kBq (10 µCi). UBT using such activity is performed routinely by the University of the Witwatersrand Scintillation Services and is ten fold higher than the 37kBq PY test® from Ballard Medical that was approved in May 1997 by the FDA (Food and Drug Administration in USA) for routine use in the USA [42, 63]. To reduce the radiation dose further, the use of even lower administered doses of 18.5 kBq (0.5 µCi) has been reported [64]. The quantity of urea in the administered dose is usually less than ten micrograms.

2.1.7 Radiation burden of the ^{14}C UBT

The effective radiation dose received with the 37kBq PY Test® test is about 3μSv [42], with the bladder receiving the highest radiation dose in both positive and negative *H. pylori* subjects, as the renal/urinary system is the major excretory route for un-hydrolysed urea. The radiation burden of this 37kBq UBT is estimated to be approximately equivalent to one day of natural background radiation [42, 58, 64] i.e. the amount of radiation every person receives from his or her surroundings (the earth, sun, food, isotopes within the body, etc.) or 1000th of an abdominal X-ray of 10mSv per radiograph.

Although the FDA concluded that there are no safety concerns and no known health risks identified from the 37kBq (1.0μCi) PY test®, legislation still prevents the UBT's general use by private nuclear practitioners in certain US states and also in other countries like South Africa and Australia.

2.1.8 UBT CO_2 kinetics

Liede-Svegborn et al [65] modelled the kinetics of $^{14}CO_2$ produced from labelled urea administered to *H. pylori* negative human subjects. According to the model, 4.6% of the administered dose will be exhaled, with more than 90% of this during the first hour after the administra-

tion. Maximum exhalation rate occurs between 10 and 20 min, decreasing thereafter exponentially with a half-life of 7min [65]. Therefore, for a 152Bq-administered dose the calculated activity of a nine mmol (1min) CO_2 exhaled breath sample at the time of maximal exhalation of an uninfected adult would be ±29 cpm disregarding the ^{14}C loss before maximal exhalation rate and assuming 100% trapping and counting efficiency. This was estimated by taking 1/7 of 2.3% of the dose (for one minute of the half-life and half of the total exhalation fraction) assuming a linear decay over the biological half-life of 7 minutes for $^{14}CO_2$ derived from urea. A method that is able to detect ^{14}C levels of 30cpm would permit distinction of *H. pylori* negative subjects at this lower dose. As *H. pylori* infected subjects exhale ten fold higher [65] concentrations of $^{14}CO_2$ of the administered ^{14}C-urea, equivalent breath activities of ±290 cpm can be expected for a 152Bq doses. According to this calculation, a clear distinction between infected and uninfected subjects with dose activities of the order of 100 to 200 Becquerel range is theoretically achievable compared to tens of thousands of Becquerel used in current tests.

2.2 Motivation for the research

The non-invasive ^{14}C-UBT with all its advantages has the drawback of requiring the administration of radioactive labelled urea. The ^{13}C UBT has the disadvantage of high cost for the isotope per test. When fur-

ther reducing the radioactivity dose and cost respectively without sacrificing test sensitivity or specificity, it would:

a) be subject to fewer (or indeed exempt from) restrictive licensing requirements by regulating authorities;

b) expose patients to even less radiation;

c) reduce the cost of the tests.

This would make the test attractive to third party payers, lower economic groups and to primary health care providers [66].

The approach of this study was to investigate:

a) reducing the radiation dose and to determine whether this would affect the specificity and sensitivity of the test,

b) whether the natural ratio of $^{13}C/^{12}C$ abundance in commercially available urea preparations could be exploited to eliminate the expensive artificially ^{13}C-labelled urea from ^{13}C-UBT,

c) theoretically and experimentally the minimum amount of ^{14}C-urea in the dose that could be detected *in vitro* (laboratory) and *in vivo* (in patients).

2.3 Major experimental components of the investigation

The research into the UBTs was conducted as four major components:

a) ^{13}C-UBT laboratory investigation,

b) ^{14}C laboratory investigation

c) ^{13}C-UBT *in vivo* investigation and

d) ^{14}C-UBT *in vivo* investigation. The laboratory investigations served to confirm the hypothesis and feasibility and establish justification for the *in vivo* investigations on human patients and controls.

CHAPTER 3

3.1 ^{13}C UBT *in vitro* investigation

3.1.1 Aim

The ^{13}C labelled urea presently used in UBT is artificially enriched with the ^{13}C isotope [37]. This enrichment is expensive and constitutes a major portion of the total cost of this test [67].

The present ^{13}C UBT study investigated the possible use of un-enriched urea. As accurate determination of low levels of ^{13}C involves high precision measurement, IRMS was selected as the method of choice. The investigation aims were therefore: 1) to determine the reproducibility and the detection limit of an extracted breath CO_2 sample using the in-house IRMS, 2) identify a suitable source of commercially available urea, 3) determine the threshold quantity of urea required.

3.1.2 Principle

The δ^{13}C value of a patient's breath and organs is typically -20‰ [68]. Using urea with a different δ^{13}C value to that of the patient's for the UBT would change the δ^{13}C of the exhaled breath. The rationale being the greater the δ^{13}C difference between the subject's δ^{13}C and the ad-

ministered urea, the greater the expected change of the $\delta^{13}C$ value from the exhaled breath.

From calculations and reasoning based on the kinetic model of Leide-Svegborne et al [65] on the *H. pylori* negative person showed that measurable change in the $\delta^{13}C$ value of the exhaled breath from a baseline $\delta^{13}C$ value is possible after administering 500mg unlabelled urea with greater than 10‰ difference and assuming a 100% conversion of the urea. For the same reason the post UBT $\delta^{13}C$ difference of a *H. pylori* positive person would be much greater.

3.2 Methods and methodology

3.2.1 Instrumentation and Chemicals

The in-house IRMS used for all the stable isotope measurements in this study was the Europa GEO Mass 20/20 with auto sampling and combustion.

Urea from the different manufacturers was weighed off with a microbalance during the $\delta^{13}C$ identification part of the project.

CO_2 separation from the breath air and the preparation of the distinctive -9.6‰ $\delta^{13}C$ CO_2 from the identified $BaCO_3$ precipitate were done on the Pyrex glass extraction system shown in Figure 4. A rotary vac-

uum pump attaining ultimate pressure of approximately 50 μmmHg was used for evacuating the system. A diffusion pump was available to improve the vacuum 50 fold but was found to be unnecessary for this type of work.

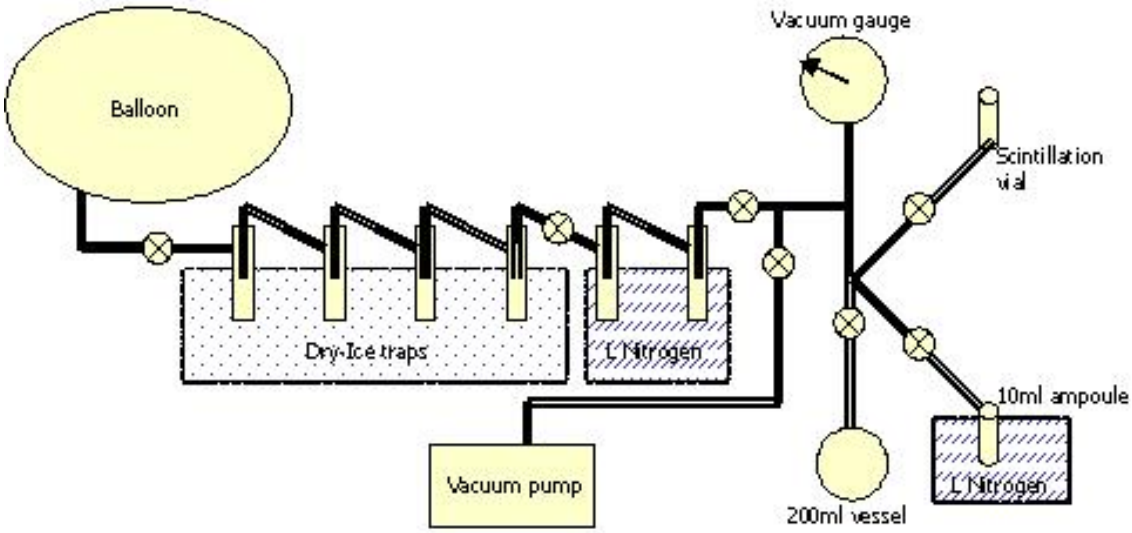

Figure 4: CO_2 extraction system

In the figure above, the breath sample contained in the balloon, enters on the left and is finally trapped on the right in the scintillation vials.

3.3 Mass spectrometry detection limit and reproducibility of CO_2 extraction

3.3.1 Methods

To simulate actual conditions of a subject performing the UBT, exhaled breath samples was collected from a volunteer in the 5 litre wine bags (Nampak, Cape Town), (Figure 8). The wine bags were then connected to the inlet of the extraction system Figure 4. The breath air was dried by passing it under vacuum through a series of traps immersed in dry-ice-alcohol slurry (-78°C) and the dry breath CO_2 was finally trapped (solidified) in the last set of traps that were immersed in liquid nitrogen (-196°C).

The CO_2 was then thawed into 10ml pre-evacuated mass spectrometer vials attached via a hypodermic needle through the flexible septum to the apparatus. After reaching one atmosphere pressure, the duplicate vials were transferred to the IRMS where the $\delta^{13}C$ levels were measured.

Repeat breath samples from the same person were obtained and prepared to evaluate the variability and reproducibility of the method.

3.3.2 Results

The average value for all 15 duplicate breath samples was 19.572‰±0.086‰ relative to PDB at 0.00‰. Figure 5 are the obtained data with the one standard deviation error bars.

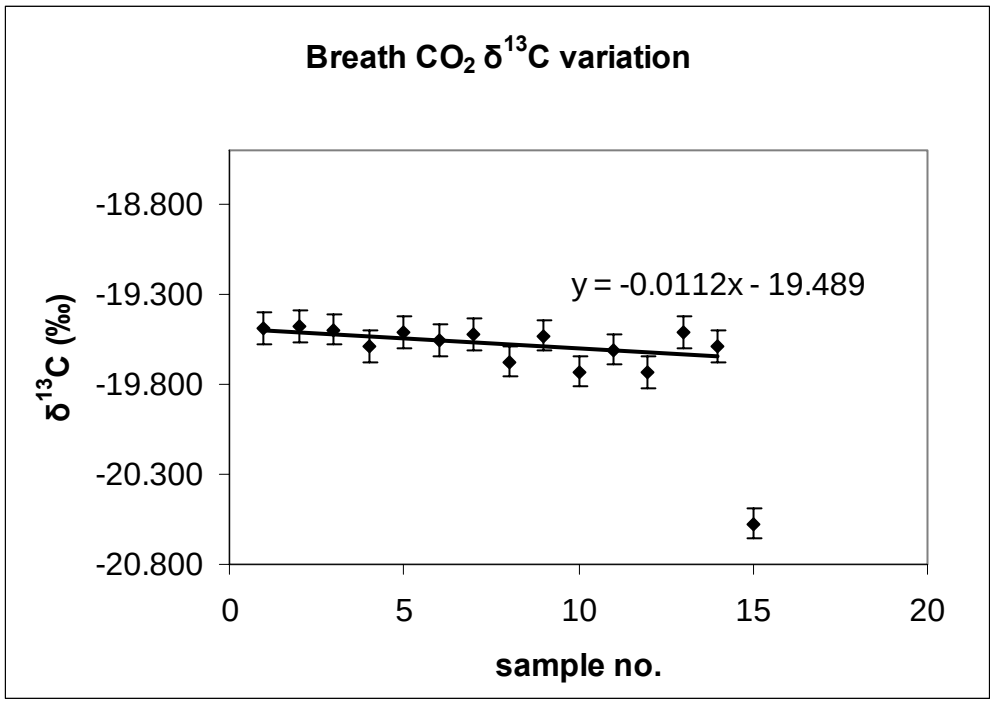

Figure 5: Sensitivity and reproducibility of breath CO_2 extraction method

3.3.3 Discussion

Reproducibility of the CO_2 extraction method is indicated by the standard deviation of 0.086‰ taken over the entire sample series. This value is factor of ten higher than reported IRMS detection limit of ±0.005‰ for routine samples. This could be attributed to a combination of factors including 1) small number of samples, 2) enhanced isotopic fractionation in the large dead spaces of the apparatus that was designed for much larger volume samples, 3) a negative slope of -0.01 across the range of the samples following the collection of breath samples over ±3 hours from a non-fasting person which could be the result of the combustion of a possible C3 plant origin meal consumed earlier [68].

Sample 15 was seen as an obvious outlier. It was collected the following day where again the diet could be the influencing factor and was therefore omitted from the statistical calculations.

3.3.4 Conclusion

The $\delta^{13}C$ measurement of repeated extracted breath CO_2 samples showed that 0.09‰ accuracy with 99% confidence was possible and thus supports its use for the unlabelled-urea ^{13}C UBT.

Variation of the absolute $\delta^{13}C$ value of the subject is less important because a pre-test baseline sample will be taken for reference of the post-test samples. A positive test is diagnosed as a significant change in the post-test $\delta^{13}C$ to the pre-test $\delta^{13}C$.

When the UBT is applied to fasting patients, the number of samples would usually be limited to less than five, and obtained within one hour, which would limit the dietary effect on the results.

3.4 Lower limit of detection of and modification of the $\delta^{13}C$ in human breath CO_2 by the addition of a discriminating $\delta^{13}C$ CO_2

3.4.1 Aims

This section of the investigation was to determine the detectability and modifying effect of adding a different but known $\delta^{13}C$ valued CO_2 on the $\delta^{13}C$ value of breath CO_2 for a dilution range. From these results, the administered threshold dose of dissimilar $\delta^{13}C$ urea for the UBT would be calculated.

3.4.2 Method

3.4.2.a Standard stock breath CO_2 preparation

Exhaled breath air from a volunteer was collected and the CO_2 extracted and isolated at 600 mmHg pressure in a 200 ml pre-evacuated vessel using the method described above.

3.4.2.b Stock 9.6‰ $\delta^{13}C$ CO_2 preparation

$BaCO_3$ precipitate identified and predetermined as being -9‰ $\delta^{13}C$ was placed in a container and connected vacuum-tight to the CO_2 extraction apparatus as shown in Figure 4. Acidifying this precipitate with phosphoric acid (non-volatile acid) liberated the CO_2 that was subsequently trapped and isolated in a pre-evacuated 200ml glass vessel at 630 mmHg pressure.

3.4.2.c Spiked range of the breath CO_2 preparation

Aliquots of these two stock CO_2 samples (breath and spike from $BaCO_3$ derived as described earlier) ranging in volumes between 0ml and 7ml were transferred to pre-evacuated 7ml mass spec ampoules using a 28G hypodermic needle. Different proportions of the two gasses were selected so as to maintain a 7ml total volume at approximately atmos-

pheric pressure (630mmHg Johannesburg, South Africa). Duplicates of each sample were then analysed with the IRMS.

3.4.3 Results

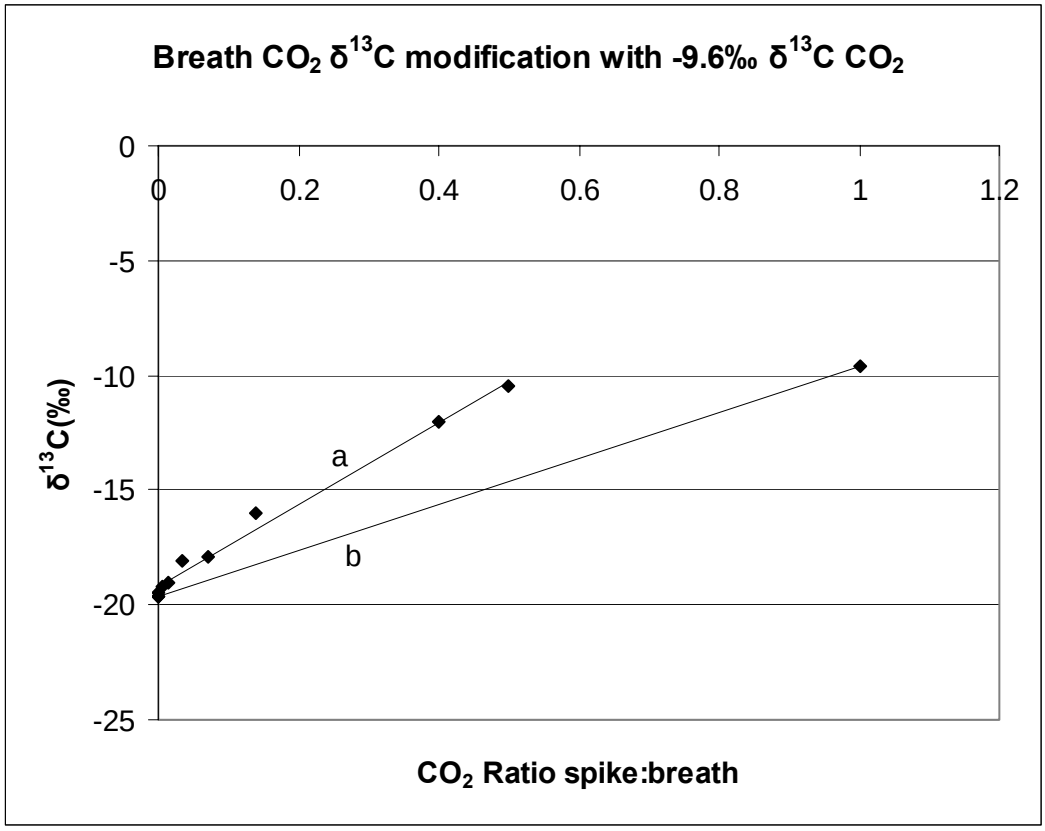

Figure 6: Graph showing the modification of breath CO_2 spiked with -9.6‰ $\delta^{13}C$ CO_2 (a) and the theoretical relationship (b)

The results shows a linear relationship (line a) in Figure 6 between tracer/breath ratio and its $\delta^{13}C$ value. However, a difference in the gradient to that of the linear theoretical mixing relationship shown as line (b) in Figure 6 is observed. This theoretical relationship is seen by connecting the data points of the pure samples.

3.4.4 Discussion

The divergence from this mixing relationship could be explained by a combination of factors, the major one being the known pressure difference between the tracer and breath samples that gave rise to proportionately more tracer being added to the mixed samples than the ratio reflects. Such a pressure difference could be due to possible air contamination of the breath sample whilst at a negative atmospheric pressure, diluting the sample during the mixing stage thus affecting the whole series of samples proportionately. Other possible factors could be the relatively crude sampling technique that was used for the transferring of small gaseous samples.

3.4.5 Conclusion

This *in-vitro* result confirm that the ^{13}C UBT without the use of ^{13}C enriched urea could be used to diagnose the *H. pylori* infection in a person provided the urea dose is at least 500mg and the $\delta^{13}C$ is more than 10‰ different from that of the breath.

3.5 $\delta^{13}C$ values of commercially available urea

3.5.1 Aim

This section of the study was aimed at identifying a suitable source of un-enriched ^{13}C urea for administration to volunteers.

3.5.2 Method

Sources of urea from different manufacturers were obtained. Six samples of approximately 100 micro grams urea were weighed off and placed in special tin sample holders. These sample holders were closed and placed in the auto combustion/sampler of the IRMS and measured employing flow through technique.

3.5.3 Results and discussion

Table 2: $\delta^{13}C$ values of urea from different manufacturers

Manufacturer	Average $\delta^{13}C$(‰)	Stdev (‰)
Kanto (Tokyo, Japan)	-61.64	1.55
Protea (Wadeville, South Africa)	-44.65	0.49
Merck (Whitehouse Station, NJ)	-30.57	0.75
Pal (Dorking, United Kingdom)	-20.51	1.20

Table 2 lists the $\delta^{13}C$ values of the urea from the different manufacturers, which range from -20‰ to -60‰. The -60‰ $\delta^{13}C$ urea from Kanto chemicals were identified as the most suitable for the using in the *in-vivo* section of the investigation being approximately 40‰ different than the average $\delta^{13}C$ values of human subjects.

Possible factors that influence the $\delta^{13}C$ values of the urea are the $\delta^{13}C$ values of the source from which the urea was manufactured and fractionation that occurred during the manufacturing process.

3.5.4 Conclusion

The urea from Kanto chemicals with the most negative $\delta^{13}C$ value are the most different from the breath $\delta^{13}C$ making it potentially suitable for use in patients. The remaining step was to apply the method to volunteer human subjects for confirmation.

3.6 *In-vivo* ^{13}C UBT with un-enriched urea

3.6.1 Aim

The objective of this study was to validate the *in-vivo* use of the Kanto un-enriched urea in the ^{13}C UBT.

3.6.2 Experimental

The investigation was run in parallel with the 7.4 kBq ^{14}C UBT study using the wine bags (Figure 7) as a method of breath collection and quantitative CO_2 extraction with apparatus shown in Figure 4

3.6.2.a Instrumentation

An adaptation to the method described in the preceding investigation is the use of the 5 litre "wine bags" (Figure 7) (Nampak flexible, Cape Town) with an attached one-way valve adaptor to collect the breath from volunteers. The stock tracer solution was prepared by dissolving 50g urea (Kanto Chemicals) in 250ml distilled water. This would permit the administration of five millilitre containing 1000mg of urea for each UBT.

3.6.2.b Sample bags

These 5 litre wine bags were chosen because they were of sufficient volume to hold 1 minute exhaled breath sample. Modified one-way CPR mouthpieces (Figure 9) were fitted onto the normal bag orifice with a silicone tube thus replacing the standard push valve that came with the bags. The "wine bags" are commercially available at low cost, durable and easy to use.



Figure 7: 5 litre wine bag left and the 1 litre PY test® Mylar bag from Tri Med®.



Figure 8: Wine bag cut open to show the laminate

3.6.2.c One-way valves

A one-way CPR (cardio pulmonary resuscitation) mouthpiece was modified by removing the polyethylene screen and heat sealing its' attachment area.

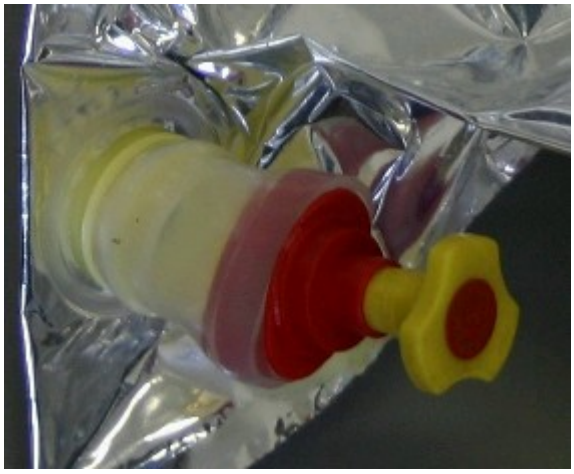

Figure 9: Attached CPR mouthpiece with sealing stopper

3.6.2.d Breath Sample collection

Subjects were instructed to hold their breath for 15 seconds before exhaling into the bag. Four exhalations per sample bag were collected. The objective was to accumulate a one-minute breath sample that should contain 9mmol (200ml) CO_2. The volunteers were each given an oral dose of 1000mg (5ml) urea (Kanto Chemicals, Japan) with -60‰ PDB $\delta^{13}C$, with 50-100ml water. Breath samples from the volunteers were taken at 0min 10min, 30min, 60min. The 0min (before urea ingestion) sample served as the reference sample.

3.6.2.e CO_2 extraction

Following the method described above in 3.3.1 and Figure 4 the CO_2 from the breath sample was extracted using an 18g hypodermic needle to pierce the silicon adaptor tube that was attached to the wine

bag in an airtight manner. The breath CO_2 was then transferred to a 7ml IRMS ampoule at one atmosphere (630mmHg) pressure and the $\delta^{13}C$ values determined with IRMS.

3.7 Results

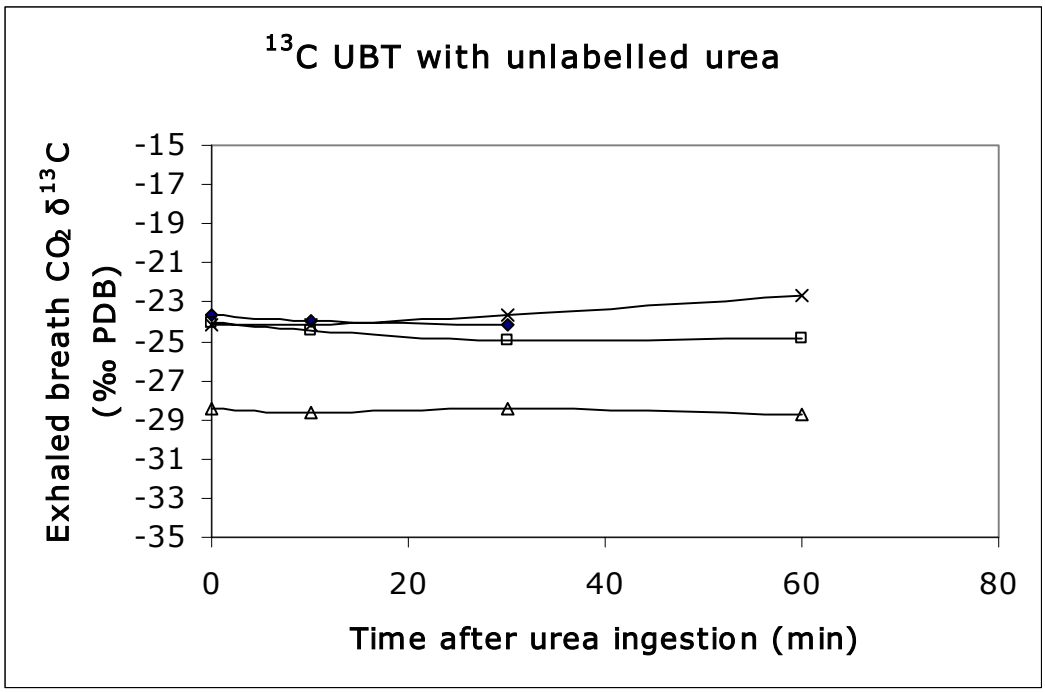

Figure 10: $\delta^{13}C$ values of exhaled breath CO_2 from separate volunteers undergoing an unlabelled ^{13}C UBT

Figure 10 above displays the $\delta^{13}C$ value of the exhaled breath after the administration of 1000mg urea with a -60‰ $\delta^{13}C$ value. The graph reflects the time depended change of the breath $\delta^{13}C$ for the first hour after ingestion.

The $\delta^{13}C$ values from three of the volunteers showed a change to a more negative $\delta^{13}C$, but the difference (-0.1‰) relative to the reference was much less than expected. Clearly missing is the well-defined and expected peak at ten minutes interval. A slightly positive $\delta^{13}C$ change was observed in one volunteer.

3.7.1 Discussion

The variation in the results observed could be ascribed to experimental/statistical error. The major factor leading to peak suppression could be urease enzyme saturation with the 1000mg urea forcing the enzyme to the V_{max} rate (V_{max} being the enzyme reaction velocity at maximal substrate concentration). This hypothesis is supported by the observed suppression (90%) and broadening of values found in the results of the parallel ^{14}C UBT (see 5.1 page 59) run.

Observed differences in the baseline $\delta^{13}C$ value between the subjects may reflect the dietary variations with individuals consuming varying amounts of C3 or C4 plant-origin material [(68)]. Indeed, one volunteer had a baseline $\delta^{13}C$ value purely in the C3 plant range whilst the rest showed mid range values between C3 and C4 and associated figure of -21‰ PDB [(68)].

The wine bags proved practical, useful, user friendly and reliable with minimal breath sample loss up to two weeks. The one-way valves

were functional but needed to be stopper sealed to prevent leakage after inflation.

The ultimate goal of replacing the enriched ^{13}C with naturally occurring ^{13}C urea for the ^{13}C UBT was not accomplished due to the insignificant results caused by the high urea dose. To overcome the saturation of the enzyme, reduction of the urea dose to 250mg may be an option. However, the probability of urease enzyme forced to saturation at V_{max} particularly in patients with low infection load still exists. This has the effect of reduced sensitivity, and a concomitant increase in false negative diagnosis.

3.7.2 Conclusion

It can be concluded that utilising un-enriched urea at 1000mg doses for the ^{13}C UBT is not a feasible option even with high sensitivity equipment and quantitative sample preparation. This implies the need to use ^{13}C-enriched urea for the UBT, to avoid the high urea dose and associated problems.

The need to redesign the CO_2 extraction apparatus to accommodate smaller breath samples was taken into consideration during this phase of the evaluation.

CHAPTER 4

4.1 ^{14}C UBT *in-vitro* investigation

4.2 Introduction

Despite the lower dose (37kBq) UBT being approved by the United States (USA) Food and Drug Administration e.g. the patented PY test® [42] (Tri-Med, Charlottesville), legislation governing its use is not uniform in the country. Private nuclear medicine practitioners are allowed to perform the test in certain states within the USA [63] whilst in others legislation prevents them from using even this dose, as is the case in other countries including South Africa. Therefore tests that utilise activity levels of ^{14}C-labelled urea below 37kBq (<1µCi) are expected to be subjected to less restrictions for general use by medical practitioners. Such usage would be in line with the accepted radiation protection principle for dose limitation of only exposing patients to radiation levels as low as can be reasonably achievable (ALARA) [69].

In South Africa, chemical substances with activity levels less than 4 kBq are exempt from licensing as radioactive substances. The IAEA (International Atomic Energy Agency) and ICRP (International Commission on Radiological Protection) exempts ^{14}C labelled chemical substances with an activity concentration of less than 10kBq/g [69, 70].

4.2.1 Theoretical

The natural background ^{14}C activity of living matter (100% Modern Carbon) is approximately 15.3 dpm per gram of carbon [53]. Assuming this activity and 100% counting efficiency, it can be estimated that a one minute breath sample of the standard man containing approximately 9 mmol CO_2 [64, 71] will have background activity around 1.65 cpm.

Improving CO_2 counting and trapping efficiency can be achieved through the use of: 1) counting on a lower background spectrometer; 2) increasing sample size by collecting larger breath samples; 3) quantifying the sample to limit absorption variation and; 4) using efficient CO_2 trapping agents; 5) quantitatively absorbing the sample. Improving and limiting the variation of the figure of merit (E^2/b) of β liquid scintillation counting, where E is defined as the counting efficiency and b the background is also very important [55]. Factors influencing the efficiency include: 1) scintillation efficiency; 2) detector efficiency; 3) quenching and 4) sample load, while these factors plus the inherent background of the spectrometer determine the background. Of note is the high scintillation and counting efficiency for ^{14}C, which is usually more than 90%.

Other factors affecting the count rates are chemoluminescence[1] and photoluminescence[2] from the scintillation cocktails that produce extra scintillations, which are detected but not associated with the ^{14}C β-radiation from the sample. Both luminescence phenomena generally lasts in the order of minutes and can be eliminated by delaying the first counting and/or by repeat counting of the samples.

Quenching reduces the count rates by firstly compressing the energy spectrum to the lower energies and reducing the counts by non-detection of the lower energy pulses particularly at high quench levels. Chemical quenching could arise from a chemical contaminant in the sample/scintillation cocktail that absorbs the β radiation form the sample, whilst colour quenching could arise form the sample colour absorbing the light generated by the scintillant [(55)]. The quench level of a sample is usually indicated by the tSIE (transformed spectral index of the external standard) or the SIS (spectral index of the sample) values, the former being a value which refers to the suppression of the spectrum from an external source where an unquenched spectrum is

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[1] Chemoluminescence is the generation of electromagnetic radiation as light by the release of energy from a chemical reaction without heat. These reactions take place within the scintillation cocktail.

[2] Photoluminescence is the property of absorbing radiant energy of one frequency and re-emitting radiant energy of lower frequency either immediately as with fluorescence or delayed between excitation and de-excitation as in phosphorescence (afterglow).

given a value of 1000. The latter is a ratio of counts for chosen windows in the sample spectrum relative to other similar samples.

4.2.2 Investigation Aim

The aim of this part of the investigation was to examine the possibility of reducing the ^{14}C dose for the UBT to exempt levels in a laboratory setting by extracting and quantitatively absorbing a defined volume of breath CO_2 into efficient trapping agents and using a low background scintillation counter for measuring the activity.

4.2.3 Experimental

4.2.3.a Chemicals and instrumentation

All CO_2 extractions were carried out using the method and apparatus (Figure 4) described in section 3.3.1 page 34. Solutions of ^{14}C urea were prepared and used for the generation of stock $^{14}CO_2$ gas.

4.2.3.b Method

Preparation of 2 litre stock unlabelled HCO_3^- (solution 1)

27g Urea and 30mg (1800U) urease were added to a 1 litre of 0.1M stock phosphate buffer (pH 8) solution to produce CO_2. The CO_2 re-

mained in solution as HCO_3^- ion by maintaining the pH level above eight.

Preparation of the 1 litre stock ^{14}C labelled HCO_3^- solution from urea with activity level of 370kBq (solution 2)

370kBq (0.2µmol) of a ^{14}C labelled urea was diluted in 10ml stock phosphate buffer before the addition of 5 mg (300U) urease. After reaction completion, the solution was further diluted 100 fold with the phosphate buffer.

Samples with projected radioactivity levels ranging from 37Bq (1pCi) to 370 Bq (10 pCi) of ^{14}C were prepared by mixing volumes ranging from 0.1ml to 1.0 ml (of Solution 2) to 40 ml (of Solution 1).

Preparation of the $^{14}CO_2$ stock and spike breath samples

^{14}C-labelled CO_2 was generated from solution 2 described above by acidifying the mixture to generate the 200ml of $^{14}CO_2$ stock solution. The gas was then thawed into a 200ml transfer bulb and removed from the system. The activity of the stock gas was obtained by adding 20ml of the gas to the scintillation cocktail and measured using the β spectrometer.

To simulate actual conditions of a subject performing the ^{14}C-UBT, the CO_2 extracted from exhaled breath samples collected over a 1-minute period from a volunteer in a wine bag was thawed into the fixed 200ml volume flask at atmospheric pressure (630 mmHg at 1800m above sea level) with the excess CO_2 vented. The isolated 200ml CO_2 sample was quantitatively transferred into the 10ml ampoule immersed in liquid nitrogen and the labelled $^{14}CO_2$ was then introduced into the 10ml ampoule via 28G hypodermic needle though a silicone connector. The spiked sample was then thawed under controlled pressure condition to allow absorption by the 5ml Carbosorb E® (Packard Bioscience BV, Gronigen, Netherland [72]) contained in the glass scintillation vial connected to the system. After the CO_2 sample had been absorbed (confirmed by a static low pressure in the scintillation vial), the vial was removed and 5ml Permafluor E® (Packard Bioscience BV, Gronigen, Netherlands [72]) scintillation fluid added. The ^{14}C activity was then measured with the low background Canberra Tri Carb 3170 liquid scintillation spectrometer.

Samples with different activity levels were obtained by spiking the fixed 200ml (9mmol) volumes of unlabelled CO_2 with volumes ranging from 0.05ml to 5.0ml of the stock of ^{14}C labelled CO_2.

Throughout the investigation, the β spectrometer counting-parameters were set for a counting time of 10 min per sample using an active 0-

30keV accumulation window as specified by the tSIE quench indicator.

4.2.4 Results

The 152Bq activity of the spiked $^{14}CO_2$ gas was determined from direct measurements of samples obtained from the stock gas.

Figure 11 shows the relationship between the recovered activity in 9mmol of CO_2, and the spiked activity levels (expressed as a percentage of the 152 Bq (0.004μCi) activity level). Each point represents the average of three sets of similarly prepared samples. From the figure, it is evident that a linear response exists from 0.02% to 1.2% of the original spiked dose. From the obtained counts, it is clear that samples with levels down 0.05% and 0.1% of the original dose could easily be differentiated.

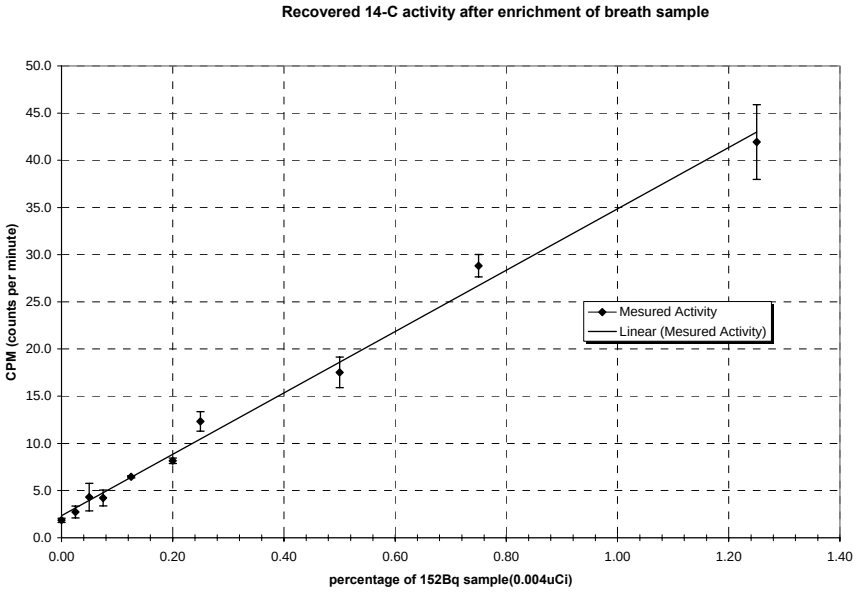

Figure 11: Recovery and detectability of ^{14}C spiked breath samples expressed as a percentage of a 152 Bq (0.004µCi) dose

4.2.5 Discussion

From the experiments it was concluded that the currently used UBT can be improved by efficient and quantitative absorption of a defined volume CO_2 into a trapping agent such as Carbosorb®, therefore allowing doses of 3.7kBq ^{14}C and less to be used in the UBT. The method was also able to reduce absorption variation in samples and improve reproducibility.

In this investigation, Carbosorb® with Permafluor E® (Packard Bioscience BV, Groningen, Netherlands) was the scintillant cocktail of choice

when compared to the commonly used hydroxy-hyamine 10-X with Instafluor®, for a number of reasons. Carbosorb® was reported to have a lower background activity and to be more efficient CO_2 trapping agent than hydroxy-hyamine or potassium hydroxide [39]. The maximum trapping level of the former is quoted [72] as being 4.8mmol/ml, whilst hydroxy-hyamine reaches CO_2 saturation at 1mmol/ml. Although Carbosorb® and hydroxy-hyamine are both alkaline solutions, hydroxy-hyamine has the added reputation of being a pesticide [53].

The need to condense the larger CO_2 sample with its scintillation cocktail into the limited 20ml volume scintillation vial dictated the choice of the higher absorption capacity Carbosorb®. In order to reduce background counts, low potassium scintillation vials were used throughout the investigation.

The use of low background spectrometers showed acceptable counting statistics with counting times of 10 minutes per sample. The accumulation window setting of 0-30keV (quench indicated) as opposed to 0-156keV, eliminated the high energy counts not associated with ^{14}C. The limit of detection with low activity levels is dependent on the counting time per sample. Results suggest that detection down to a limit of 0.1 cpm can be achieved by increasing the counting time.

4.2.6 Conclusions

The above investigation validated the method of quantitative absorption of a defined volume CO_2 into Carbosorb®/Permafluor® and suggests a means for reducing the activity of the ^{14}C labelled urea dose to less than the 3.7kBq in patients undergoing the ^{14}C-UBT.

The experiments support the use of these low doses to *in-vivo* investigations, described in the following chapter.

CHAPTER 5

5.1 *In-vivo* "exempt" ^{14}C UBT investigation

5.1.1 Aim

The aim was a) to develop a protocol and to verify the use of low (<3.7kBq) doses of ^{14}C-urea in humans for the UBT, b) confirm the biological elimination rate of the Leide-Svegborn model for *H. pylori* negative persons c) apply this protocol to verify whether it is sensitive enough for discrimination between *H. pylori* negative and positive persons and d) identify the time of maximal tracer exhalation.

5.1.2 Introduction

This *in-vivo* investigation selected adult patients with confirmed *H. pylori* infection status by the ClOtest® rapid urease test and volunteer control subjects of unknown *H. pylori* infection status. The *H. pylori* positive patients were all gastroscopically diagnosed to have PUD and/or gastritis while certain of the control subjects had a history of PUD and treatment.

Each of the subjects were given two ^{14}C UBT consisting of the "exempted 7.4kBq" followed by a 330kBq (9μCi) dose, 1μCi less than the

standard routine 370kBq (10μCi) ^{14}C UBT which served as a control for this study.

The terms, specific activity and activity concentration are often used interchangeably resulting in frequent confusions in interpretation. In line with the IAEA terminology [69, 70], the definitions of the terms "activity concentration" and "specific activity" are as follows: specific activity refers to the activity per unit mass of the nuclide and activity concentration refers to the activity of the nuclide relative to the matrix of which it is a part.

5.1.3 Experimental

5.1.3.a ^{14}C Tracer preparation

The standard 370kBq labelled urea have a specific activity of 2GBq/mmol of urea. This part of the study first investigated the addition of 50g unlabelled urea to the 370kBq urea with the aim of satisfying the current exempt level of 10kBq/g ICRP, however the level is interpreted [69] (see 5.1.7.a page 67). This investigation was carried out concurrently with the *in vivo* ^{13}C UBT investigation (see 3.6 page 42) with the 1000mg unlabelled urea, having a specific activity of 7kBq/g of carbon.

5.1.3.b Conducting the 7.4 kBq (0.2μCi) ^{14}C UBT and the standard 370kBq ^{14}C UBT

As described in 3.6.2.d, breath samples were collected at 0 min, 10 min, 30 min and 60 min after administration of the exempt level 7.4kBq/1000mg urea (5ml of the stock solution). After completion of the 7.4kBq UBT, the standard 370kBq at 330kBq was administered to the same person as a control. The "exempt" samples were counted on the Packard TR/SL 3170 spectrometer while the standard UBT samples were counted in the Packard TR 1660 spectrometer.

5.1.4 Results of the 0.2µCi/1g-urea UBT

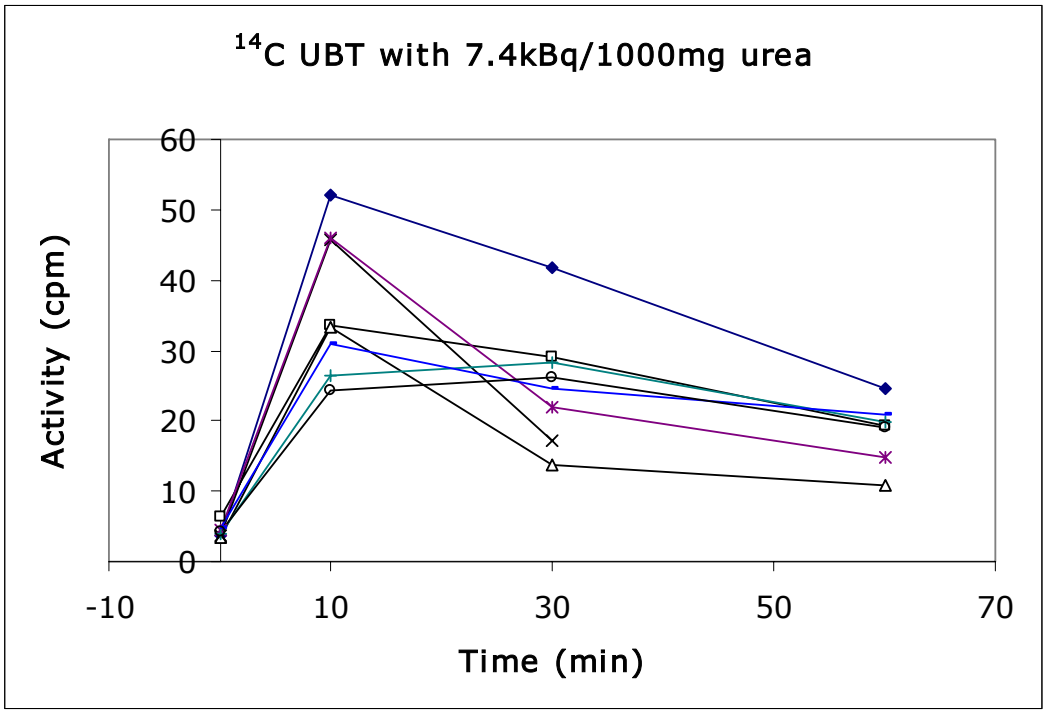

Figure 12: ^{14}C UBT with high urea load of 1000mg

Figure 12 shows the breath ^{14}C activity with time after the administration of 7.4kBq ^{14}C labelled 1000mg urea. Although the results show peak values at ± 10 minutes, no discrimination is observed between *H. pylori* positive and negative persons as was determined by the parallel run 370kBq UBT [39]. The activity levels were also much lower than expected [73].

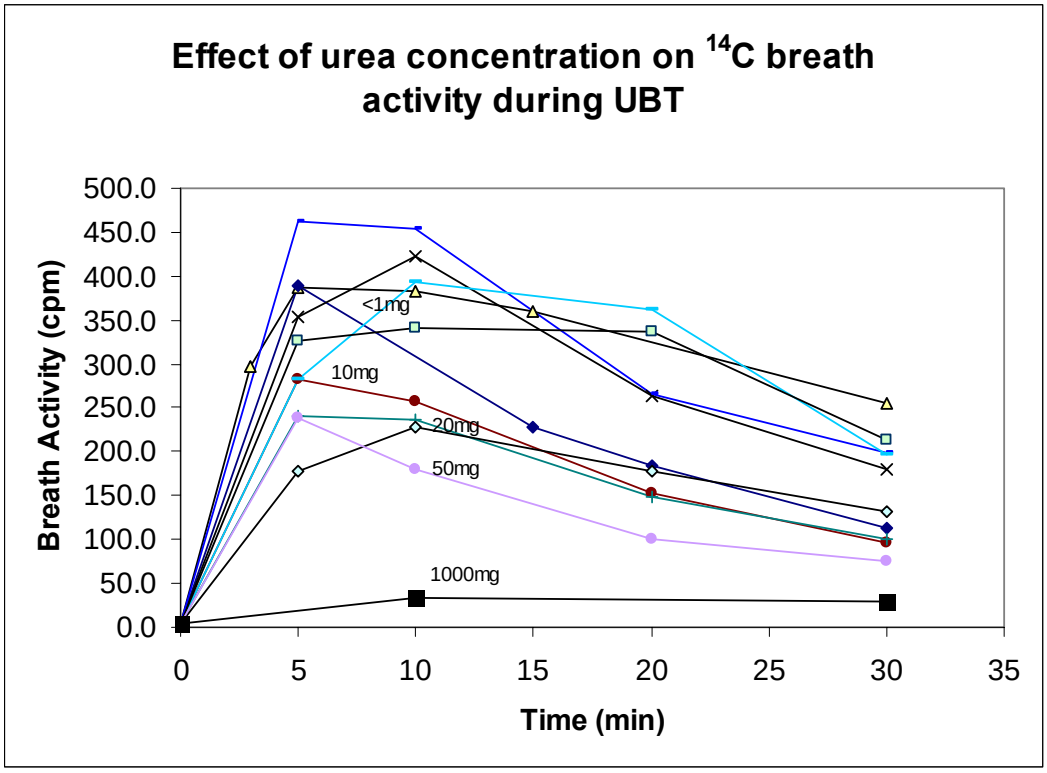

Figure 13: Urea dose effect on the breath activity with the "exempt" ^{14}C UBT

Figure 13 shows the breath ^{14}C activity-time relationship of an *H. pylori* positive person following the administration of varying doses of non-radioactive urea added. The plot with the lowest activity was from a sample with the most urea added (1000mg) whereas activity increased with lower doses of administered urea.

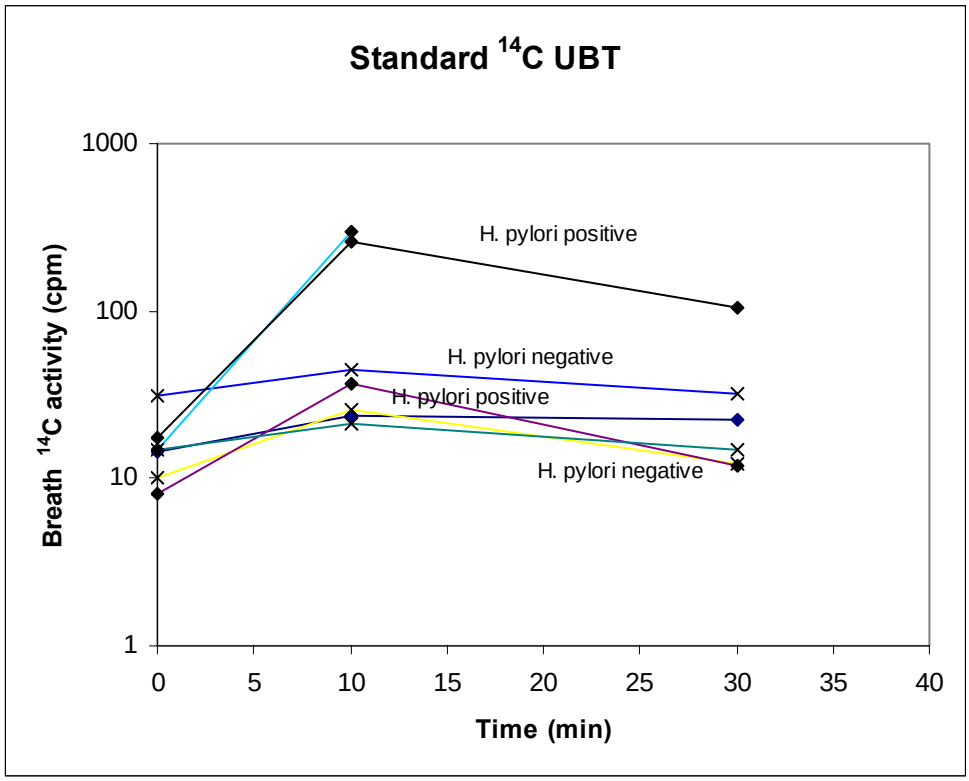

Figure 14: Breath activity of the standard 330kBq ^{14}C UBT

Figure 14 above is a time-activity graph of the breath from patients and controls using the standard 330kBq ^{14}C UBT showing the distinction between *H. pylori* infection positive and negative subjects.

5.1.5 Discussion

The lower sensitivity and higher background of the TR/SL1660 spectrometer resulted in the baseline counts for the standard 330kBq UBT varying in the range of 10 to 20 cpm with positive sample values between 25 and 400 cpm. Shown in Figure 14 is a semi-logarithmic plot of the breath ^{14}C activity with time for the standard UBT. Using this

protocol, positive diagnosis is made when the 10-minute count rate is double or higher than the baseline values. In this study, 10-minute samples had activity levels in the order of tens of cpm range for most of the subjects except two. The highly positive *H. pylori* patients gave results with maxima in the hundreds of cpm. The lower values could be ascribed to the trapping capability and saturation of the hyamine, which may have lead to lower sensitivity and specificity.

By comparison, the more sensitive and lower background TR/SL 3170 spectrometer used for the exempted UBT (Figure 12 and Figure 13) showed reproducible baseline results to within 0.5 cpm even with ten minutes counting times.

As hypothesised in section 3.7.1 (page 47) the 1000mg urea used may have saturated the urease into V_{max} (see Figure 13). The associated zero-order kinetics with prolonged conversion of the radioactive urea could be an explanation of the lower and non-discriminate peaks of Figure 12. With this dose the elimination half-live was estimated to be approximately sixty minutes, which is eight times longer than the predicted seven minutes by the Liede-Svegborn model.

During preliminary investigations, variations in the volumes of samples collected were noted in the CO_2 pressure during quantification of the extraction procedure. Instead of the needed 1min CO_2 sample, wine bags were often filled with three or four breaths within 15 seconds.

Subsequently, the method of collecting breath samples was standardised by requesting the subjects to hold the breath for 15 seconds before exhaling into the bag. By repeating the procedure four times, an effective one-minute breath sample was collected. This was confirmed by a higher-pressure reading in the same fixed volume.

The bag collection method was found to eliminate possible accidental subject exposure to such harmful chemicals as the alkaline trapping solutions. Further advantages include the ease of transport and storage. Samples can be stored for at least one week before analysis. This method was acceptable and preferred by patients and volunteers alike.

5.1.6 Conclusion

It can be concluded from this preliminary investigation that the addition of excess non-radioactive urea to reduce the activity concentration of the administered labelled urea, was the major reason for the failure of this preliminary UBT protocol. Higher specific activity urea is required to obtain satisfactory/suitable signal (Figure 13).

Following the above conclusion, an investigation into the use of lower ^{14}C UBT dose, but with no further addition of non-radioactive urea, is described below.

5.1.7 Revised protocol with 3.7kBq (0.1μCi) ^{14}C UBT without the further addition of non-radioactive urea

5.1.7.a Introduction

For this part of the study, instead of adding non-radioactive urea to the 7.4kBq ^{14}C labelled urea, 370kBq ^{14}C labelled urea was diluted with 100ml water to give an activity concentration of 3.7kBq per gram of water. A reason for the 3.7kBq UBT choice was to comply with the South African regulations, which states that a substance is regarded as non-radioactive when activity is less than 4kBq. Furthermore, the 3.7kBq per gram of water activity concentration is in line with the IAEA exempt level for activity of nuclide relative to the matrix, (section 5.1.2).

5.1.7.b Experimental

The 3.7kBq administration dose was prepared by dilution of the 370kBq with 100ml water and 1ml aliquots dispensed into Eppendorff vials and frozen until required. Each subject were administered 3.7kBq (1ml) labelled urea with 50ml water taken immediately after. This was to ensure that all the urea reached the stomach and to limit its incidental conversion in the mouth.

Breath samples were collected in the manner described earlier but the number of sample collections were increased and were taken at intervals of 0 min, 5 min, 10 min, 20 min, and 30 min. This was done to identify the time of maximum tracer exhalation as preliminary results showed the 10min sample to be the most active. For some volunteers, sample collections were extended to 24 hours to determine the longer-term breath elimination of the urea.

Experience gained during the previous sections of the investigation identified the need to redesign the CO_2 extraction apparatus to accommodate smaller volume breath samples used during the UBT.

The requirements identified for a redesigned CO_2 extraction system were:

1) reduction of the large dead volumes of the large bore tubes,

2) simplification of the gas volume-measuring step by incorporating the set volume in the traps,

3) portable and compact,

4) user friendly, practical and robust.

Revised CO_2 extraction apparatus

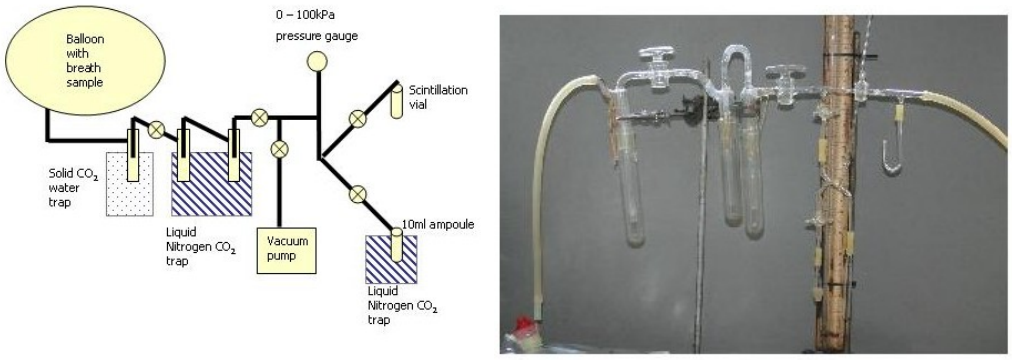

Figure 15: Diagram and photograph of the simplified CO_2 extraction apparatus

To satisfy some of the criteria in the list, a revised extraction apparatus using redundant Pyrex® glass tubes and traps was built. Fragility of glass forced the use of flexible silicon pressure tubing at sections where high movement was expected such as bag-, pump-, and scintillation vial attachments. The vacuum pump used is a single stage rotary vacuum pump. Pressure measurements were carried out using a mercury manometer.

This modified apparatus provided higher sample throughput without cross contamination between samples. This was confirmed by preparing and measuring zero percent modern carbon samples at random between higher activity test samples.

5.1.8 Results of the 0.1µCi UBT

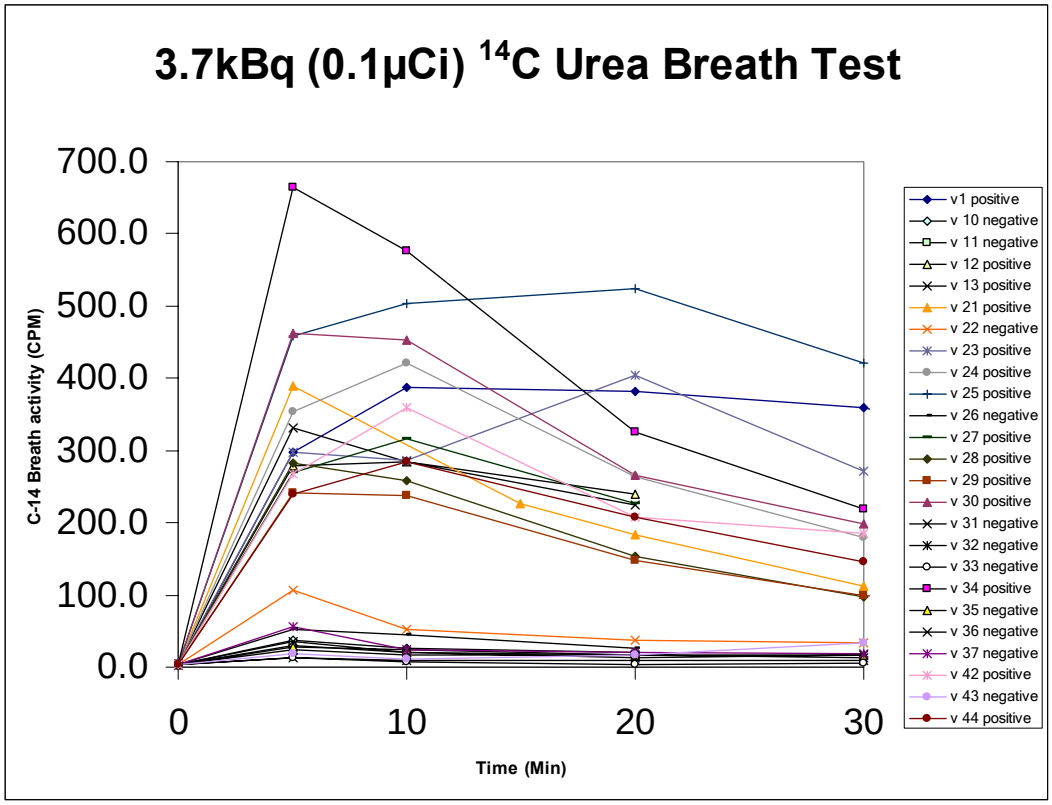

Figure 16: 3.7kBq UBT showing the individual time-breath activity over 30 minutes after the urea administration

Figure 16 shows the activity of the exhaled breath air for 30 minutes after the administration of 3.7kBq ^{14}C labelled urea to individuals who took part in the investigation. Two distinct bands of activity are noted corresponding to and thus discriminating between positive and negative *H. pylori* infection status of the individual.

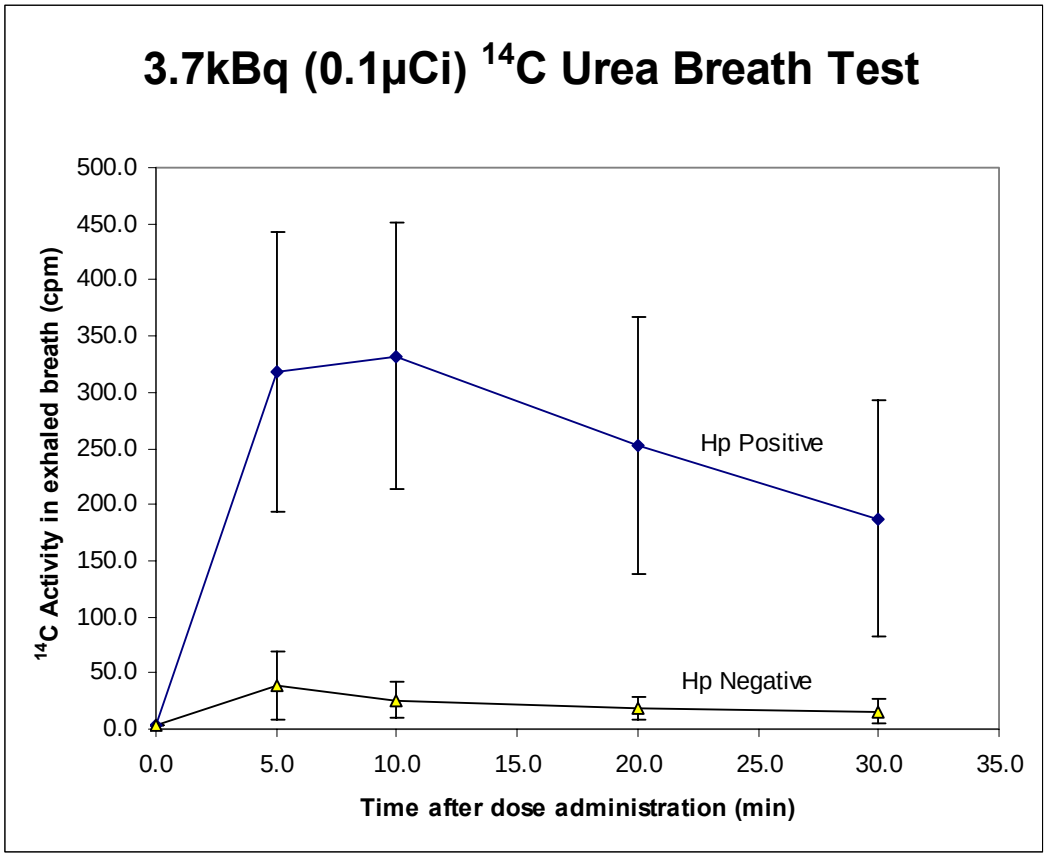

Figure 17: Graph of the averaged counts with one standard deviation error bars for the 3.7kBq UBT-data from Figure 16

Figure 17 shows the averaged results for the ^{14}C activity in the breath of the subject having taken the 3.7kBq UBT.

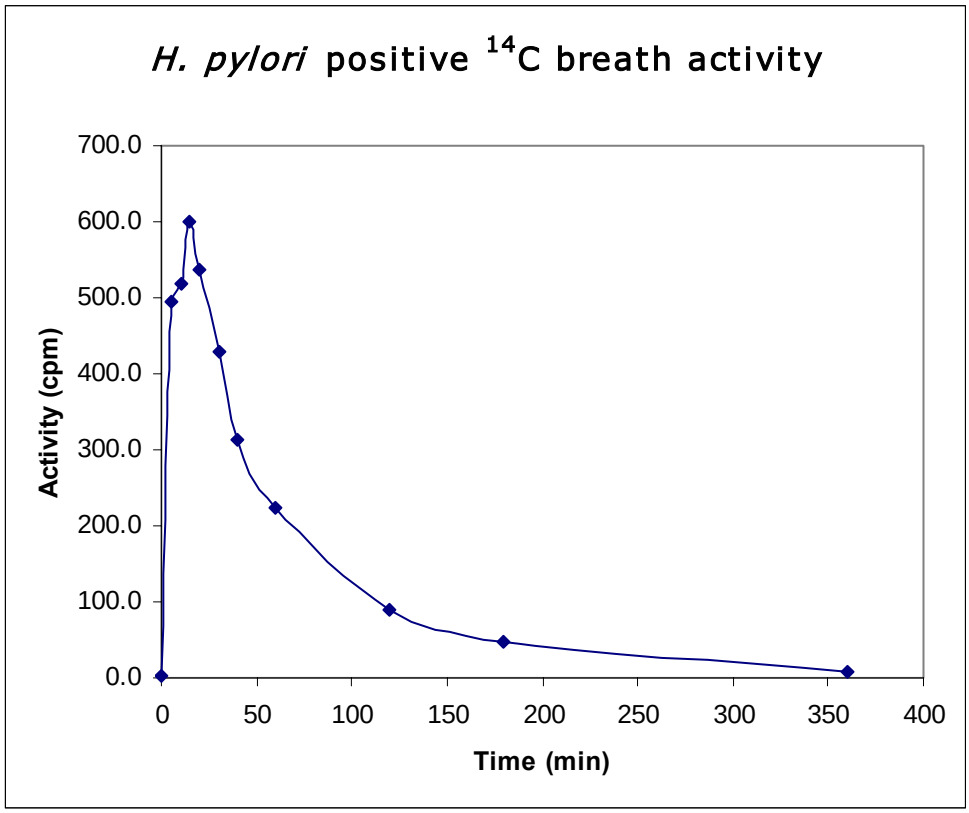

Figure 18: $^{14}CO_2$ breath activity for an *H. pylori* positive person after taking 3.7kBq ^{14}C labelled urea during the UBT

Figure 18 shows the breath activity of a *H. pylori* infected person for six hours post urea administration.

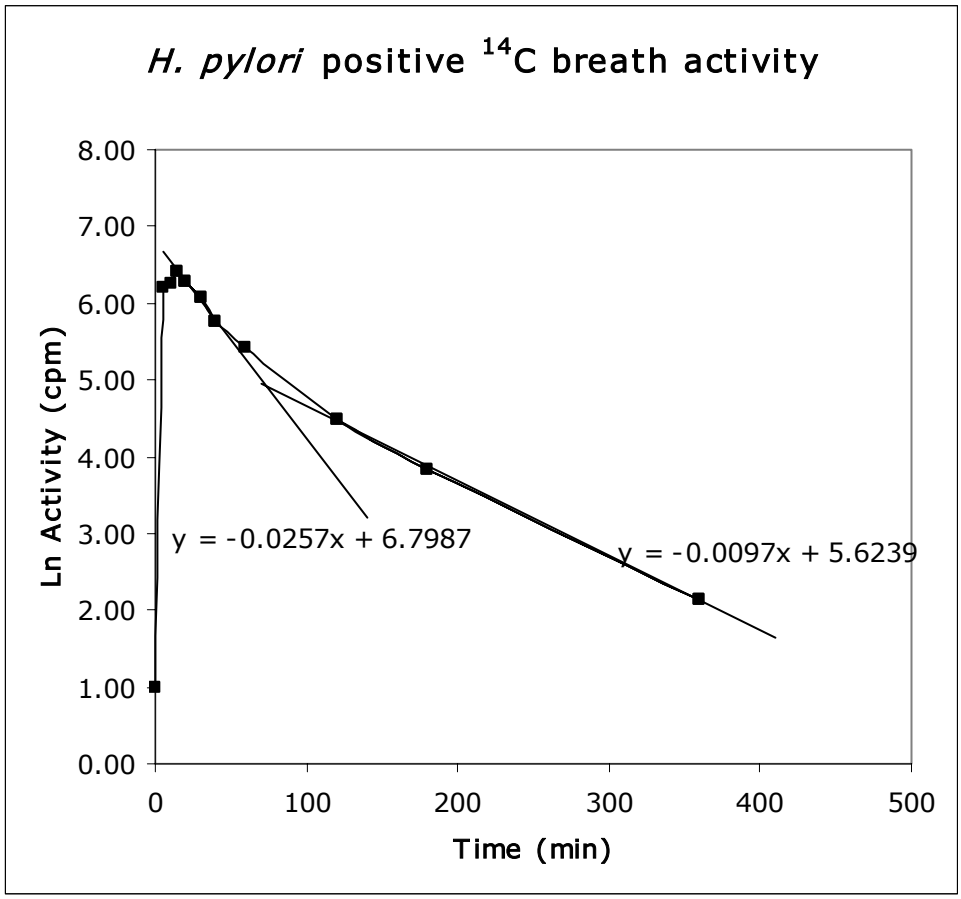

Figure 19: Semi-logarithmic plot of the activity-time data for Figure 18 of the breath $^{14}CO_2$ from an *H. pylori* positive person

Figure 19 display the data from Figure 18 on a semi-logarithmic graph, showing two distinct exponential elimination rates followed during the first six hours. ^{14}C removal rate from the breath had a half-life of approximately twenty minutes during the first hour, followed by an elimination rate with one-hour half-life for up to six hours.

5.1.9 Discussion

The main finding of this experiment was the two distinct bands that represent either the *H. pylori* infection positive or negative subjects respectively (Figure 16 and Figure 17). The results were confirmed by either the rapid urease test or the standard 370kBq (10µCi) UBT. No intermediate results were obtained suggesting the 100% sensitivity and specificity of this UBT protocol.

Both *H. pylori* positive and negative persons showed maximum count rates at approximately 10 minutes with activities of the positive persons about 10 times higher than those of the negative persons.

During the study, UBT was repeated on the same volunteers after about two weeks. A progressive increase of the count rates for equivalent timed samples were observed, some showing an early and often high activity in the breath five minutes after administration (Figure 21 page 82). This was particularly evident in formerly tested *H. pylori* negative persons, now approaching the activity levels of the infected persons. Such peaks are reported to be caused by *H. pylori* and other urease-producing bacteria in the mouth, which could be reduced by the brushing of teeth before taking the UBT [(74)]. However, brushing teeth prior to the UBT did not reduce the counts and auto hydrolysis of the urea before administration was assumed as the most

likely cause. Indeed, preparation of fresh urea eliminated these early peaks supporting the possibility of auto-hydrolysis in the unfrozen urea solution. The appearance of the early peaks is explained by the liberation and absorption of the labelled CO_2 when the alkaline solution (urea, NH_4^+, HCO_3^-) enters the acidic stomach environment.

Care should be taken by using fresh or frozen urea to prevent the effect of auto-hydrolysis as it could lead to false positive results.

This UBT protocol produced consistent and reproducible baseline sample count rates of 1.5cpm ± 0.5cpm, equivalent to one hundred percent modern carbon. This suggests that counting of a baseline sample could be excluded when using a sensitive instrument like the Tri-Carb TR/SL 3170 and the diagnosis of *H. pylori* infection status could be made on one sample taken at ten to fifteen-minutes after urea administration. The five-minute samples were also able to discriminate *H. pylori* positive from *H. pylori* negative. However, the results may be influenced by other factors like urease-producing bacteria in the mouth [(74)], auto-hydrolysis of the urea (see above), thus increasing the uncertainty of the results.

A few of the subjects had a light meal within two to four hours before taking the UBT but this did not seem to influence the results. However, it was preferred if the subjects were fasted before taking the UBT. During the sample collection, eructation into the bags after the

^{14}C was administered, would add $^{14}CO_2$ directly from the stomach into the bag and would lead to results which may not reflect labelled CO_2 in the breath.

The elimination rate for the $^{14}CO_2$ from the breath was estimated with a half-life of approximately 20minutes for the first hour for *H. pylori* positive (see Figure 19) and approximately 15 minutes for *H. pylori* negative persons (data not shown). This is of the same order to that of the Leide-Svegborn model [65] although their model was based on *H. pylori* negative subjects and a higher ^{14}C urea activity was administered.

Supporting the sensitivity of this method are results of a few trial samples with 370Bq (0.01µCi) administered urea which could still discriminate between positive and negative *H. pylori* infection (data not shown).

Comparative results (see Appendix 2) between the TR3170 and the less sensitive and less expensive TR1660 scintillation counters showed that the latter could also be used for counting the low dose ^{14}C UBT. However, baseline sample counting may be necessary, as the inherent background of such a spectrometer is less stable and higher at approximately 15cpm. Furthermore, the use of the complete 0-156keV ^{14}C channel is recommended.

5.1.10 Conclusion

The results suggest the protocol described for the ^{14}C UBT with 3.7kBq (0.1µCi) activity can be used to diagnose the *H. pylori* infection status of a person and that one sample taken between ten and fifteen minutes after the oral administration is needed. The baseline sample is not required and a cut-off activity value of 100cpm of the 10-minute sample would permit discrimination between *H. pylori* positive and negative subjects.

Brushing of the teeth before taking the UBT was found not to be necessary, however care must be taken to prevent urea auto hydrolysis before administration as this could lead to false positive results.

CHAPTER 6

6.1 General conclusion

The possible use of ^{13}C isotopically labelled urea being replaced with commercial available un-enriched urea in the ^{13}C UBT for the diagnosis of *H. pylori* was systematically investigated. Despite positive *in-vitro* results, the major reason for the failure of the *in-vivo* protocol lay in the large dose of urea (1000mg) administered, saturating the urease enzyme, (zero order kinetics) extending the time and reducing the signal peak that might have been observed. It was concluded that some enrichment of the ^{13}C is necessary or less urea be used. Further experimental work is required to verify this hypothesis.

The investigation of 3.7kBq exempt level of ^{14}C labelled urea showed positive results. Sensitivity and specificity appeared to be almost 100%, excluding known factors that may give rise to false negative results such as recent ulcer treatment and or antibiotic treatment.

The method of collecting the breath samples in wine bags and quantitatively extracting and absorbing a defined volume of the breath CO_2 using the specifically developed apparatus are believed to be the contributing factors that gave rise to these results. The routine use of the protocol however awaits the outcome from a clinical trial.

The sensitivity and specificity of the protocol adopted for the ^{14}C UBT indicated that the diagnosis of *H. pylori* infection was possible from a single sample taken between ten and fifteen minutes after oral administration of 3.7kBq (0.1 μCi) ^{14}C labelled urea. Such a protocol is expected to reduce laboratory and overall cost of the UBT further.

In summary, the method offers such advantages as: 1) exemption from licensing allowing safe/unrestricted use, 2) a substantial cost saving, 3) reduction of radiation exposure to patients to less than one hour of background radiation and 4) ease of sample collection with minimal patient discomfort; i.e. the disadvantages of presently used 10μCi UBT, method.

Further refinement of the CO_2 extraction apparatus such as sturdiness and appearance are needed for commercial exploitation.

Appendix 1

Patent

Geyer J.A., Nam T.L., Candy G.P. Improvements in the measurement of ^{14}C in CO_2. SA Provisional Patent 2004/4893 (2004).

Publication

Geyer J.A., Nam T.L., Candy G.P. (2004). Modified ^{14}C urea breath test for the detection of *Helicobacter pylori* using exempt levels of ^{14}C. Submitted for publication in Journal of Nuclear Medicine Technology.

Appendix 2

Other preliminary and related experiments

A 2.1 Comparison of spectrometers

^{14}C activity from different enrichments and comparison between counters

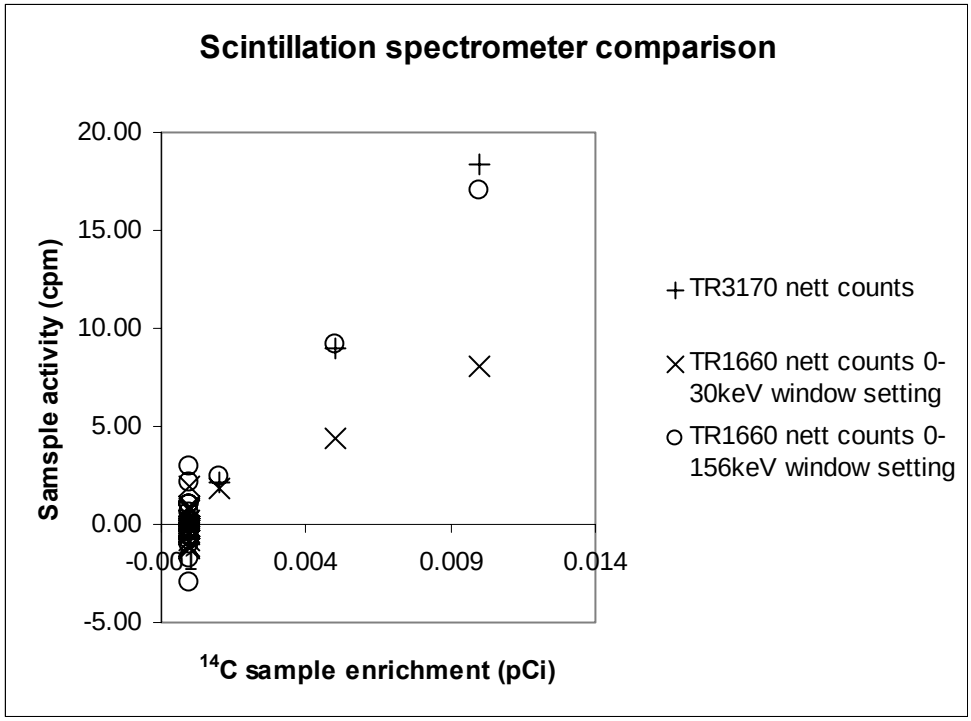

Figure 20: Comparison between the low background Packard TR3170 SL and the TR1660 SL scintillation spectrometers

The graph above shows the correlation between two spectrometers. Measuring the samples in the TR1660 on a wider window (0-156keV)

improved the lower limit of detection to very similar values to that from the low level TR3170 spectrometer.

A 2.2 Auto hydrolysis of urea before administration

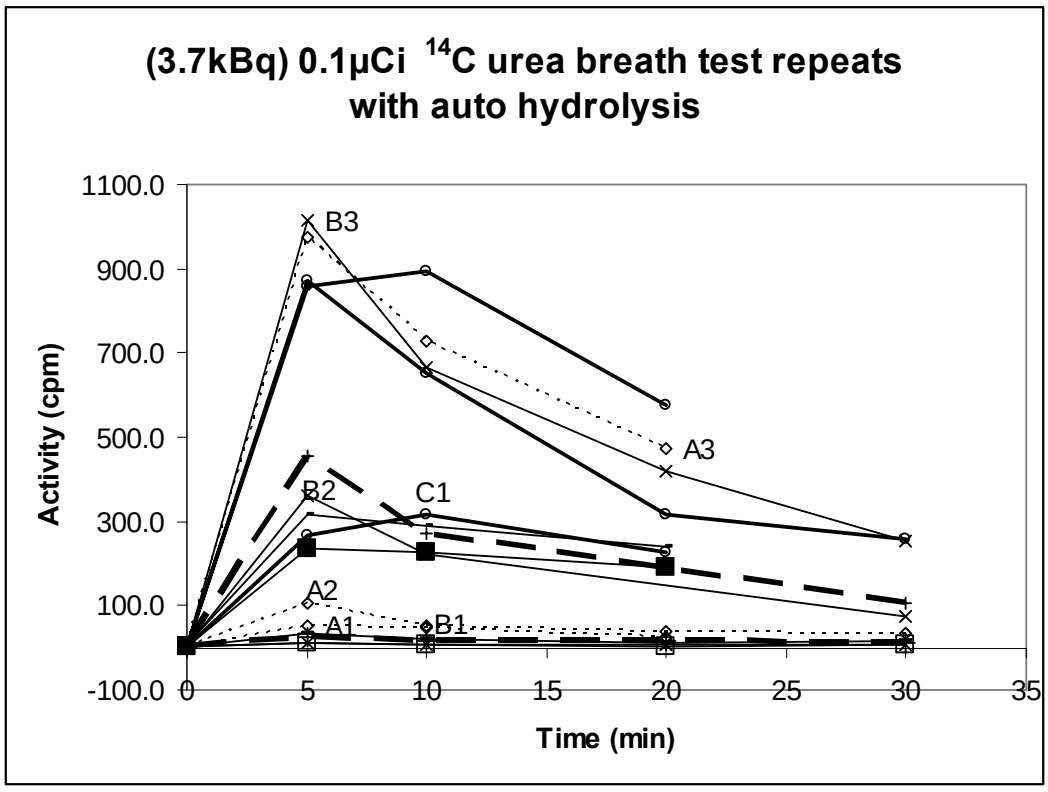

Figure 21: Time-activity graph of repeated 3.7kBq UBT for six individuals showing urea auto-hydrolysis.

Figure 21 above shows the data of the repeated 3.7kBq UBT in six subjects. Plot A1 represent the initial CO_2 sample breath activity of a *H. pylori* negative person. Plot A2 represent the first repeat sample and A1 the second repeat sample. Similarly B1, B2 and B3 represent the breath activities for the initial, first- and second repeats for another *H.*

pylori negative individual. Plot C1 represent the initial activity of an *H. pylori* positive person.

Shown in the plot is the increase in the breath activity of the repeat UBT's samples, with early (5min) peaks. The subsequent samples for the *H. pylori* negative persons (A2, A3, B2, B3) had activity values equivalent to those from a *H. pylori* infection positive person (C1). This would result in false positive diagnosis. Evidence presented in section 5.1.9, suggested auto-hydrolysis of the pre-administered urea as the probable cause.

Ethics clearance certificate

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