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MICRODOSE ^{14}C-UREA BREATH TEST IN DETECTION OF *HELICOBACTER PYLORI*

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Etiologic role for *Helicobacter pylori* (Hp) seems to be well established in gastric pathology. The high urease activity of Hp can be used to detect this bacterium by non-invasive urea breath tests (UBT). We validated the microdose version of the test in which 37 kBq ^{14}C-urea is given orally in capsule. With the cut off value >100 DPM as positive, UBT results correlated highly significant with combined results for invasive methods i.e. CLOtest + histology score. The reproducibility of the test was 100%. The results obtained for the breath test performed locally were almost identical with that read at remote laboratory. The data found for fasting and fed states of subjects agreed in 87%. When ^{14}C-urea was confined in the mouth of both Hp positive and Hp negative patients UBT showed the presence of urease activity in the mouth cavity. ^{14}C-urea capsule based breath test is highly reliable, safe, and reproducible for detection of Hp in the stomach. Results can be obtained within 15 min if a scintilation counter is nearby, or breath samples can be mailed to a testing laboratory for analysis.

Key words: *^{14}C-urea breath test, Helicobacter pylori*

INTRODUCTION

Over one hundred years after microscope observation by Jaworski (in 1886) of spiral-shaped bacterium *Vibrio rugula* in human stomach (1) and twelve years after the original isolation and rediscovery of this curved bacilli from gastric biopsies by Warren and Marshall (2) called today *Helicobacter pylori* (Hp), we know that this interesting bacterium infects over half of the worlds population (3). Type B gastritis (non-autoimmune), inflamed gastric mucosa, is well known to be associated with Hp infection (4). After more than a decade of studies, Hp has gained acceptance as the most important factor in the pathogenesis of peptic ulceration (5). Hp a gram-negative bacterium may be

found predominantly on the luminal aspects of the gastric epithelium beneath the mucus layer and in the gastric pits (6). The mode of transmission is probably oral-oral or fecal-oral. Its prevalence increases in lower socio-economic class people, particularly in developing countries and increases with age (7, 8). The role of Hp in non-ulcer dyspepsia is still controversial, and its association with increased gastric cancer risk offers an exciting opportunity for further research. Antibiotic treatment, which eradicates Hp infection, may cure peptic ulcer disease and is recommended by the National Institute of Health (9). A fundamental principle for specific antimicrobial therapy is the accurate diagnosis.

The "gold standard" method for detection of Hp includes rapid urease test and histology of endoscopic mucosal biopsies (10, 11). This requires an invasive technique (endoscopy of mucosal biopsy) followed by Campylobacter-like Organism (CLO) test and histological (e.g. Giemsa stain) evaluation or culture of biopsy specimen. In addition, polymerase chain reaction (PCR) can identify and distinguish Hp from other helicobacters (12). Hp may also be detected non-invasively by either urea breath test (UBT) using ^{13}C or ^{14}C-urea (13) or serology based on enzyme linked immunosorbent assay (ELISA). Serological testing may detect IgG or IgA antibody to Hp in patients with Hp infection (14) but since antibodies take many months to be cleared out from circulation after eradication of Hp these tests may not be useful to confirm factual Hp status e.g. immediately after Hp eradication theraphy.

The UBT test is the most specific, non-invasive way of detecting Hp. Urease is not present in mammalian cells, so the presence of urease in the stomach is an evidence that bacterium is present though, the connection between gastric urease and gastric spiral bacteria was not made until 1984 (15). Before the rediscovery of Hp in the stomach by Warren and Marshall (2), it was believed that gastric urease was produced by the mucosal cells . In order to detect gastric urease, urea labeled with ^{13}C or ^{14}C is swallowed by the patient. If urease originating from the Hp is present in the stomach, urea is split to form bicarbonate and ammonia at the interface between the gastric epithelium and lumen. The HCO_3^- soon enters the bloodstream where it is carried to the lungs and rapidly expired as $^{13}CO_2$ or $^{14}CO_2$.

^{14}C-urea breath test has been used since 1987 when first attempts were made to treat peptic ulcer patients for Hp infection. The original test system described by Marshall and Surveyor (16) employed 370 kBq dose of ^{14}C-urea solution which created some problems related to the form and dosage of the isotope and the collection of breath samples (17) . In this paper an enhanced test in which the micro-dose of ^{14}C-urea enclosed in quick dissolved capsule is evaluated. The aims of this study were therefore: 1. to evaluate a microdose (37 kBq) ^{14}C-urea enclosed in a quick dissolve test capsule; 2. to assess whether the fasting period is required before the procedure of ^{14}C-UBT; 3. to see if

breath sample results changed when they were mailed to a remote site for analysis; 4. to define the diagnostic ranges of ^{14}C-UBT for Hp-positive and Hp-negative patients. 5. to estimate possible influence of oral urease on UBT results in situations when labeled urea was in confined in mouth cavity.

MATERIALS AND METHODS

The study was approved by the University Ethical Committee and all patients signed informed consent. Patient Hp-status was evaluated by a gold standard of histology (any curved bacteria in antral or corpus biopsy sample was recognized as Hp-positive) and by rapid urease test CLOtest (Campylobacter-like Organism test, Delta West Pty Ltd, Bentlley, Western Australia Ltd). CLOtest result was assigned as Hp-positive when a change of color from yellow to red was immediate or 3h after adding the biopsy sample on the slide containing urea and phenol red at 30—40°C. When CLOtest remained yellow and histology was negative the Hp status was considered negative. 159 consenting patients who met the entry criteria for the study (not pregnant women, age between 18 and 75 years, not subjected to gastric resection, and were not after medications such as bismuth or antibiotics taken at least in the past 4 weeks and/or sucralfate or omeprazole taken in the past 2 weeks) were given a 37 kBq capsule of ^{14}C-urea (manufactured by TriMed lab in Charlottesville, Virginia, USA) with 25 ml water and an additional 25 ml of water 3 minutes later. Breath samples (1 mmol CO_2) were collected in benzothonium (Hyamine) hydroxide in methanol (1 mmol Hyamine hydroxide made up to 2.5 ml in methanol with bromothymol blue as pH indicator, hereafter called *collection fluid*) at baseline and then at 5 min intervals for 30 min. The patients blew into a drinking straw through a non-return collection trap into the collection vials. Two extra breath samples were collected in aluminized balloons at times 10, 15, 20 minutes (6 balloons samples in total). Some subjects were invited to return within one week to have further breath test after eating breakfast (two rolls with butter and jam, a cup of coffee or tea). The fasting breath test (F ^{14}C-UBT) was compared with non-fasting test (NF ^{14}C-UBT) and gold standards in the same subjects. On the same day as the test, one set of breath samples was transferred from balloons to collection fluid at the study site. The second set was sent to TRI-Med Specialties in Charlottesville, Virginia where was analyzed no sooner than 96 hours after test. Separate group of 24 subjects were tested twice (at one day interval) in order to estimate the reproducibility of the test.

In a group of 27 Hp-positive or Hp-negative subjects (according to ^{14}C-UBT), the oral rapid urease activity test was performed using CLOtest (Jatrox-Hp-test, Proctor and Gamble, Weiterstadt, Germany) by dissolving in distilled water urea plus phenol red and adding to this solution the saliva, dental plague and material obtained from gingival pockets. In addition to that, 10 ml of water solution containing 18.5 kBq ^{14}C-urea and phenol red as a volume recovery marker was taken and kept (without swallowing) in the mouth by each patient for 5 min. During this period, while keeping the solution with ^{14}C-urea in the oral cavity, each studied patient was asked to inflate at one min interval plastic bags through his nose (nasal collection of breath sample). That was followed by spitting entrapped ^{14}C-urea solution, washing out the mouth and finally proceeding ^{14}C-UBT as described above by collecting the breath samples at 7, 10, 15, 20, 25 and 30 min time points from the start of experiment. The spitted fluid was used to estimate the recovery factor.

After breath sample was transferred into collection fluid, the scintillation cocktail was added and the sample was counted for 5 minutes in β-counter (LKB 1211 model) calibrated before with calibration standard of ^{14}C-hexadecan of know activity. The zero time point sample was used as background and was subtracted from each result. The final results were expressed in

disintegrations per minute (DPM) by counting at the same day a known standard sample and dividing CPM results by the machine efficiency (usually 0.85). These parameters give an accuracy (± 0.16 SEM) of $\pm 13\%$ at 50 DPM and $\pm 3\%$ at 1000 DPM.

In subjects, in whom the results obtained with ^{14}C-urea were compared to golden standard the gastroduodenoscopy was performed and two antral biopsy samples were taken from the antral mucosa for histology and one sample for rapid urease CLO-test using plastic slides with urea and phenol red. Histology samples were fixed in formalin, embedded in wax, sectioned in routine fashion and stained with Giemsa at Pathology Department. Presence of curved or spiral organisms on gastric epithelium was graded as follows: 0 = none, 1 = difficult to find but definite curved organisms, 2 = easily found curved organisms in several parts of the specimen, 3 = many curved organisms throughout the specimen (massive colonization). By adding histology grade (0—3) to the result of CLOtest (0 for negative, 1 for positive) a total score ranging 0 to 4 could be obtained for each subject tested. The relationship between the amount of the radioactivity (in DPM) in the exhaled air (10 minutes after ^{14}C-urea capsule ingestion) and the combined score of CLOtest (0—1) plus histology (0—3) was evaluated.

Statistical significance and analysis were determined with the paired t test and performed using Microsoft Exel 5.0.

RESULTS

For the results obtained from Hp-positive and Hp-negative patients at 10 min, the proposed cutoff value of $\geqslant 100$ DPM correlated significantly with combined CLOtest and histology results. The range 50—99 DPM was considered as undefined and results below 50 DPM as definitely negative. The results of breath test and combined results constructed by adding histology grade (0—3) to the result of the CLOtest (0—1) in the same subjects correlated highly giving a total scores ranging from 0 to 4 (*Fig. 1*). In this manner only Hp—positive patients are considered since nearly all Hp-negative patients gave histology grades and DPM near zero. There was no or very little overlap between grade 1—2 and grade 3—4. The breath test tended to be more strongly positive in patients with massive mucosal colonization with Hp, grades 3—4 (histology grade 2 or 3 and CLO test 1). For grades 1—2 Hp colonization of Hp was found to be patchy or sparse. The relation was highly significant ($p < 0.001$, R-sq. = 0.281). Breath samples collected and read immediately at our laboratory were almost identical to those sent to Tri-Med (Charlottesville, VA, USA) and read at no earlier than 96 hours (*Fig. 2*). The diagnostic accuracy of each single sample read blind at Tri-Med was the same for all samples sent. In Hp-positive subjects the $^{14}CO_2$ excretion peak occurred between 10 and 15 minutes so that a single sample taken between these two time points gave optimal diagnostic accuracy. *Table I* shows $^{14}CO_2$ recovery at 15 min after ^{14}C-urea capsule ingestion in 24 patients (17 Hp-positive and 7 Hp-negative) in whom the test was repeated on two consecutive days. The reproducibility was very good (100%) , there were some day-to-day not significant variations but no patient was reclassified.

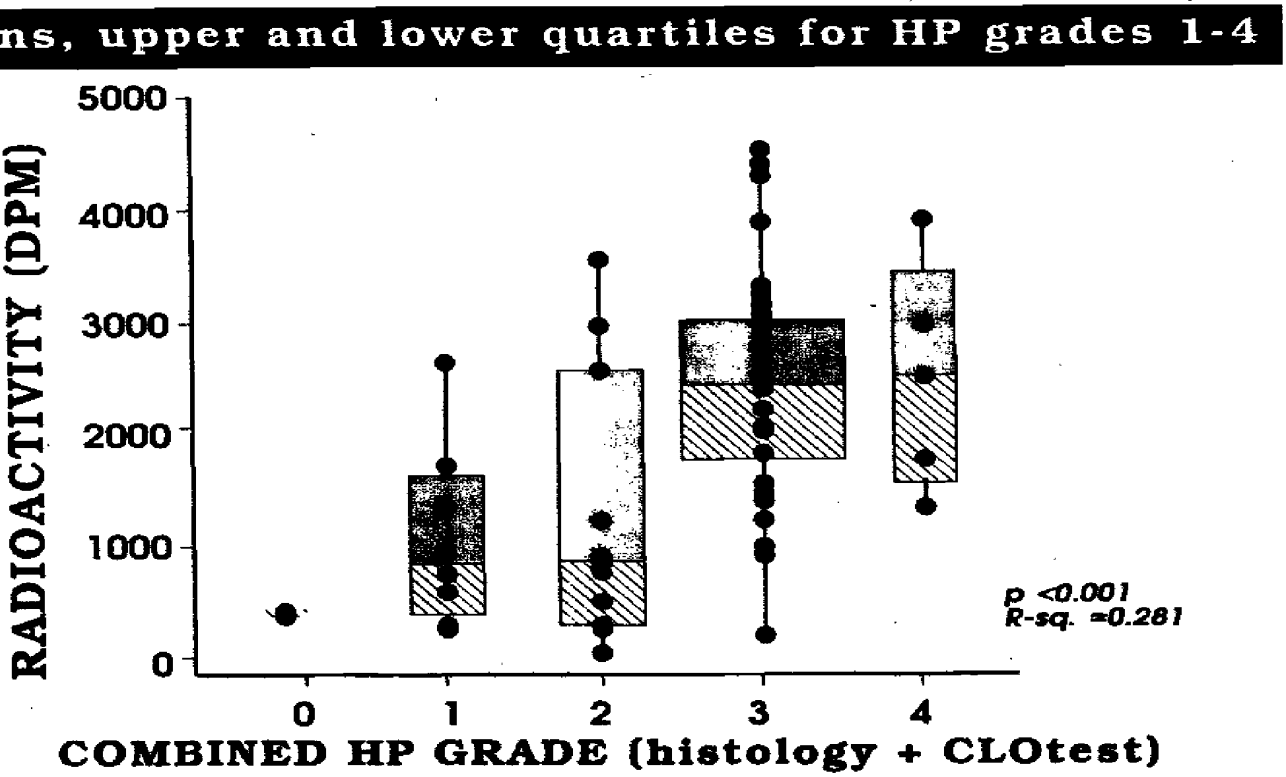

Fig. 1. Means, upper and lower quartiles for Hp grades 1—4. Note: Boxplots show means, upper and lower quartiles; circles show location of individuals. Only Hp-positive patients are shown. Hp grade was defined by summing histology and CLOtest results from initial histological review. Thus, zero grade was a patient in whom both tests were negative initially but in whom review of histology revealed small number of Hp. Grade 4 was a patient in whom histology grade was 3 and CLOtest was positive (1).

Table 1. The reproducibility of the ^{14}C-UBT. $^{14}CO_2$ recovery was measured for each patient twice (at one day interval) in the breath samples exhaled 15 minutes after ^{14}C-urea ingestion.

Patient status	Day 1 dpm, (15 min)	Day 2 dpm, (15 min)
HP+(16)	1086±70	1069±575
HP− (8)	15±8.3	17 ±9.1

Breath test results presented on *Fig. 3* were obtained from patients (9 Hp-positive) tested after an overnight fast and on the next day, 30 minutes after breakfast. Generally, the positivity of breath test was not affected by eating, though the DPM values were significantly decreased during first 15 min after food intake and peak values in fed patients were somewhat delayed.

Radioactivity in breath samples obtained after liquid ^{14}C-urea solution (18.5 kBq, in 10 ml) was confined for 5 min in the mouth are presented on the *Fig. 4.* Both Hp-positive and Hp-negative (by usual ^{14}C-UTB) patients showed urease activity originated exclusively from the mouth cavity. The maximum of $^{14}CO_2$

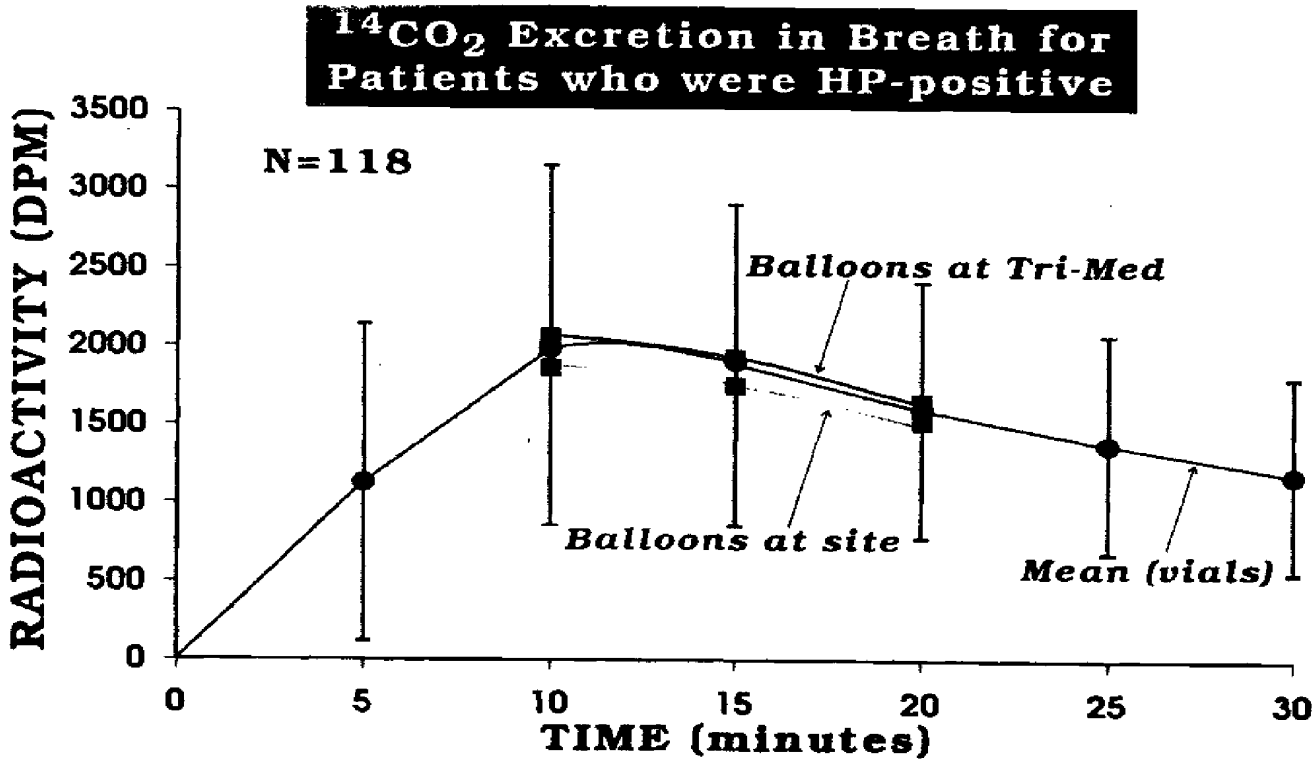

Fig. 2. ^{14}C excretion in breath for patients who were Hp-positive. Note: Major curve from 0 to 30 min shows mean for Hp-positive samples collected directly into vials and read at the study site (seven time points were available for vials). Secondary lines represent values of three samples collected into balloons and read at the study site (dotted) or at Tri-Med Specialties in Charlotesville, USA (thin solid line) no earlier than 96 hours after collection.

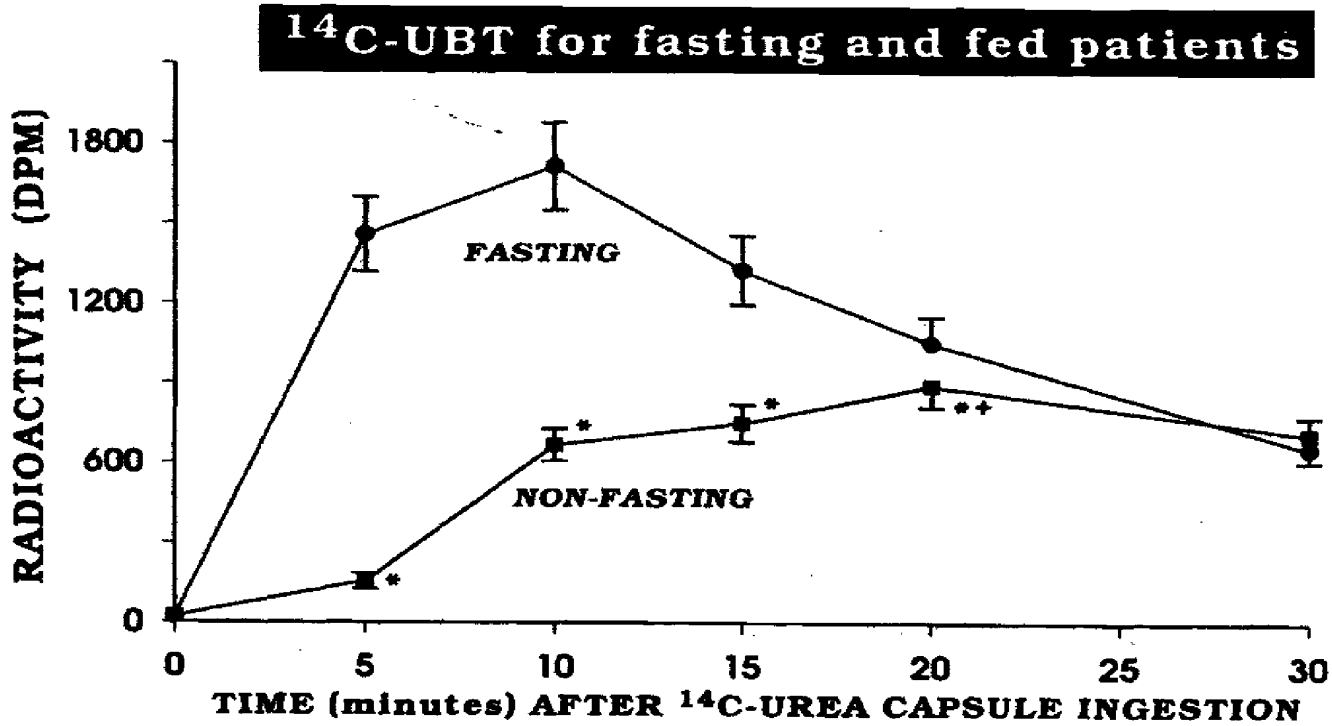

Fig. 3. Breath test results for Hp-positive volunteers after overnight fast and on the next day, 30 minutes after breakfast. Asterix idicates significant decrease ($p < 0.05$) below the value obtained at the same time intervals in fasting subjects. Cross indicates significant decrease in peak radioactivity in fed subjects as compared to the same subjects fasted overnight.

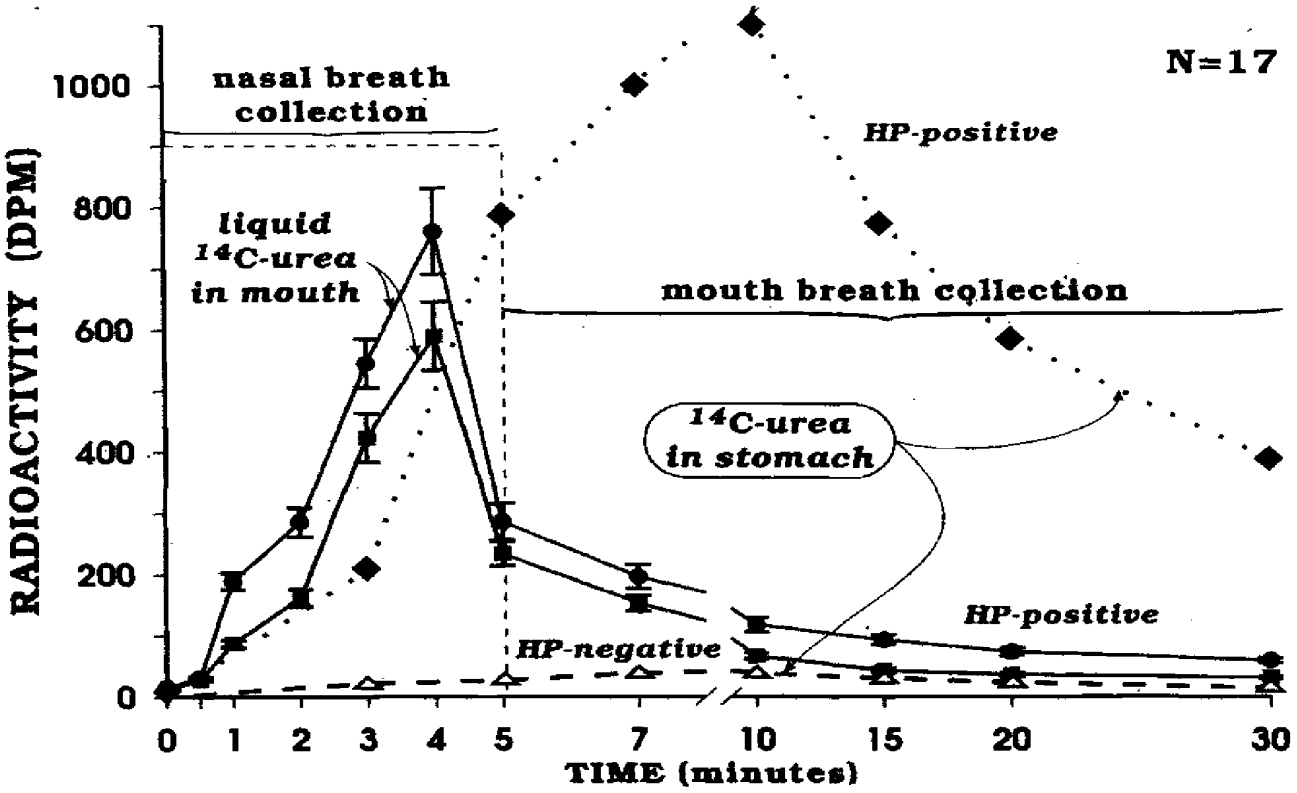

Fig. 4. Radioactivity in breath samples obtained after liquid ^{14}C-urea (18.5 kBq) was confined for 5 minutes in the patient's oral cavity. Note: At 0.5, 1, 2, 3, 4 and 5 minutes breath samples were collected trough nose (nasal collection). The breath samples at 7, 10, 15, 20, 25, and 30 were taken orally after the mouth was washed out. Typical profile for gastric UBT (after capsule ingestion) are represented by dotted and broken lines respectively.

expired during 5 min nasal breath collection was observed between third and fourth min after the start of experiment overlapping the excretion profile (broken line) of the gastric ^{14}C urea test in the Hp-positive subjects. No adverse effects were caused by the breath test.

DISCUSSION

The use of micro-dose (37 kBq) ^{14}C-urea enclosed in easy dissolving capsule is highly sensitive, accurate and very convenient test for the diagnosis of Hp infection of stomach. In this study, a breath sample collected at 10 minutes detected Hp with 99,5% sensitivity. By adding histology grade to the results of CLO-test the highly positive correlation was found between the DPM at 10 min and combined histology + CLO-test scores as recently reported by Marshall (17). Since, the ^{14}C-urea is not exposed to mouth urease and possible oral or oropharynx contamination with urease containing bacteria such as *Streptococus salivarius or Actinomyces viscous* (18) is greatly reduced, variation in Hp-negative subjects (near background level of ^{14}C) is much less and improved discrimination between Hp-positive and Hp-negative subjects can be

achieved. The separation of Hp-negative and Hp-positive is far greater than with liquid rapid ^{14}C-urea breath test. Therefore, the test can be done with a lower dose of isotope i.e. with only 37 kBq of ^{14}C-urea. The capsule test also allows simple handling procedures with no significant risk of isotope loss or spillage.

Our results are fully comparable with those obtained by Marshall et al (17) who first introduced microdose (37 kBq) capsule ^{14}C-UBT. Various urea test protocols recommend additional stages and precautions, such as the use of the mouth washing with citric acid solution to supppress oral urease activity (19, 20), preliminary meal to delay gastric emptying (21), and/or the addition of a "cold" urea substrate to saturate the urea enzyme system (22).

The theoretical potential risk of false positivity (at 10 min) due to buccal urease activity was reported earlier by Raju et al (23) who also used microdose (37 kBq) of ^{14}C-urea administered in liquid form. Our results obtained with ^{14}C-urea confined exclusively to the mouth show (*Fig. 4*) that these precaution can be avoided without loss of diagnostic accuracy in a case when solid ^{14}C-urea in capsule was used. Therefore, for routine work the use of microdose capsule-based ^{14}C UBT and breath sample collection at 10 min has similar diagnostic accuracy eliminating the problem of possible false-positive results in early period breath samples. Additional advantage of micro-dose ^{14}C-UBT in capsule is speed of obtaining results and far lower cost than that for ^{13}C-UBT. Final result can be obtained within an hour (including the "trapping" of $^{14}CO_2$ into the benzothonium hydroxide and counting in β-counter), thus allowing physician decisions to be made at the same day of clinic visit.

The earlier data obtained by Marshall et al (16) as well as data from others show that nearly all of the ingested isotope is excreted either in urine or in breath within the next 72 hours so that radiation exposure from the test is negligible (24, 25). The only drawback of ^{14}C-urea is the fact that it is radioactive and therefore, is a subject to regulations concerning its use. However, with the current microdose method, the amount of isotope is so small that the test actually gives less exposure than normal people receive from background radiation in one day (0.3 mrem/day) and is hundreds of times less than accepted procedures such as mammograms or upper gastrointestinal x-ray series (26).

The major and original finding of this paper is clear demonstration that meal based ^{14}C-UBT in the same subject is equally sensitive as that without earlier meal ingestion. The only difference in ^{14}C-UBT without ingestion of meal, as compared to that with meal, is the significantly higher radioactivity in the former test recovered in first 15 min after administration of ^{14}C-urea containing capsule. The peak of radioactivity after meal was delayed, occurring at 20 min upon the swallowing of ^{14}C-urea capsule, and was significantly lower than that obtained with that capsule in fasting subjects. This difference in the

amount of $^{14}CO_2$ in exhaled breath samples between tests performed in fasting and non fasting subjects could be simply attributed to the dilution of the ^{14}C-urea by larger volume of gastric content after meal as compared to that in fasting stomach. The fact that the radioactivity peak after ^{14}C-urea in fed subjects was significantly lower and delayed in time as compared to that in fasted subjects militate against the usefulness of the meal based breath test presumably because the ^{14}C-urea cannot easily be rapidly emptied from the stomach. If anything, ^{14}C-UBT in fed subjects gives less pronounced but delayed peak in $^{14}CO_2$-related radioactivity of exhaled air and if possible should be avoided in routine ^{14}C-UBT.

In conclusion, the microdose capsule (37 kBq) ^{14}C-UBT test has proved to be a convenient, non-invasive test and safe for detection of gastric Hp infection with accuracy equal to invasive methods such as histology and rapid urease test. Results can be obtained within 10—15 minutes if counting instrumentation is available at site or mailed to a remote testing laboratory for analysis. The test with ^{14}C-urea formulated in capsule performed in fasting state is as accurate in diagnosis as that carried out after a meal. Oral administration of liquid ^{14}C-urea should be avoided because it may lead to a false positive result due to possible presence of bacteria with urease activity in the oral cavity.

Finally, it seems that the urea breath test as non-invasive diagnostic method offers powerful and usefulness technique in evaluation of anti microbial therapy (as the method of choice for follow-up the Hp eradication of *Helicobacter pylori*) as well as convenient method for the epidemiological studies on the Hp prevalence.

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