JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 1996, 47, 3, 545—553

Rapid communication

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NEW APPROACH TO ^{13}C-UREA BREATH TEST: CAPSULE-BASED MODIFICATION WITH LOW-DOSE OF ^{13}C-UREA IN THE DIAGNOSIS OF *HELICOBACTER PYLORI* INFECTION

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This study was designed to evaluate a novel modification of the non-invasive capsule-based ^{13}C-urea breath test (^{13}C-UBT). 114 patients were tested for *Helicobacter pylori* (HP) infection with the use of only 38 mg ^{13}C-urea administrated in solid capsulated form. Obtained results were compared with tissue based methods: histology and rapid urease test (CLOtest). Results of histology and/or CLOtest were considered as the gold standard for each patient. In addition, also capsule-based, micro-dose (37kBq) ^{14}C-urea breath test (^{14}C-UBT) was performed. With a cut-off for delta-over-base values of 5‰ (i.e., 5 per mil), ^{13}C-UBT results (measured by non-dispersive infrared spectroscopy, NDIRS) correlated highly significant with combined results for invasive methods i.e., CLOtest + histology score. Compared with histology, CLOtest, and the gold standard, the diagnostic values of the test were: sensitivity 97%, specificity 95%, with positive and negative predictive values about 90% and 98% respectively. The modified ^{13}C-UBT test was found to be in full concordance with ^{14}C-UBT; there was 100% agreement in the diagnostic classification of all positive (89) and negative (25) patients. Described modification of ^{13}C-UBT showed that presented modification of ^{13}C-UBT is an excellent, simple, low cost, non invasive, and safe diagnostic tool in HP detection and should be recommended particularly in cases when the use of radioactive urea is contraindicated.

Key words: *Helicobacter pylori, ^{13}C-urea breath test, ^{14}C-urea breath test*

INTRODUCTION

Helicobacter pylori (HP) one of the commonest bacterial infection, may lead to gastritis, peptic ulceration, non-ulcer dyspepsia and possibly gastric cancer (1, 2). Eradication of HP infection may lead to long-term remission of peptic ulceration and improvement of gastritis (3). It is obvious, therefore, that the test detecting the bacterium is of great clinical significance. A practical and available alternative to endoscopy with tissue-based method (culture, histology,

and rapid urease test: CLOtest) are serology or non-invasive techniques. Urea breath tests with either ^{13}C-urea or ^{14}C-urea offer unique advantage as noninvasive, very sensitive, fast and highly specific methods by comparison with laborious culture and histology. UBTs can detect HP colonization within the stomach assessing the entire mucosa non-invasively avoiding the risk of sampling error or an observer bias. In contrast to serology, UBT offers excellent diagnostic value as a post treatment control to confirm eradication of HP (4).

The possibility of non-invasive detection of HP using the ^{13}C-urea breath test (^{13}C-UBT), (which was more costly than ^{14}C-UBT and needed mass spectrometry), was first reported by Graham et al. (5). Nevertheless, the method with the use of stable ^{13}C carbon isotope has been promoted on the basis of safety because radiation exposure could be avoided. Since then, several modifications of the test procedure have been described. All of them employed different relatively large amounts of expensive ^{13}C-urea (75-150 mg) administrated orally in a liquid form with or without previous meal (6). Such way of urea ingestion may lead to false positive results due to possible contact with oral or oropharynx cavity, where the presence of other urease-producing bacteria is very likely (7, 8). To overcome these drawbacks, we propose in this study a new modification of ^{13}C-UBT in which low-dose ^{13}C-urea was enclosed in quick dissolved gelatin capsule. The aim of this study was, therefore, to evaluate the usefulness of a minidose (38 mg) ^{13}C-urea enclosed in a quick dissolve test capsule for HP detection. Thank to such urea administration the problem of false-positive results in early breath sample was omitted, there was no need for a test meal, the test time and costs of diagnosis were reduced.

MATERIALS AND METHODS

Subjects

114 patients attending for routine upper gastrointestinal endoscopy entered the study, which was approved by the University Ethical Committee. All of them signed informed consent. The exclusion criteria for the study were; possible pregnancy, age under 18 years, previous gastric surgery, recent use of medications such as bismuth or antibiotics (within 1 month) and/or sucralfate, proton pump inhibitors or histamin (H_2)-receptor antagonists taken in the past 2 weeks. For each examination patients were asked to come after overnight fast.

^{14}C-Urea Breath Test

The breath tests were performed immediately prior to the endoscopy or the next morning as described previously (7). Briefly, all consenting patients were given a 37 kBq of ^{14}C-urea in capsule with 25 cc water and an additional 25 cc of water 3 minutes later. Breath samples (1 mmol CO_2)

were collected in benzothonium (Hyamine) hydroxide in ethanol (1 mmol Hyamine hydroxide made up to 2.5 cc in ethanol with bromothymol blue as pH indicator, hereafter called collection fluid) at baseline and then at 10, 15, 20 and 30 min later. After breath sample was transferred into collection fluid, the scintillation cocktail was added. The sample was left undisturbed until autoscintilation became negligible and then 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 (± 2 SD) of $\pm 13\%$ at 50 DPM and $\pm 3\%$ at 1000 DPM. A positive test was an increase over baseline of >150 DPN in any sample. The range 100—150 DPM was considered as undefined (non diagnostic), whereas a negative test required all samples to be <100 DPM.

Capsule-based ^{13}C-Urea Breath Test

The same group of the patients as above was tested at random order non-invasively for the presence of HP also with the use of ^{13}C-urea (MassTrace Inc., Mass, USA). After overnight fast breath sample was collected from each patient (prior to urea administration) at baseline. This was followed by ingestion of gelatin capsule containing 38 mg of ^{13}C-urea with 25 cc of water. After 3 min additional 25 cc of water was drunk by the patient and breath samples were collected at 10, 15, 20 and 30 min later. Breath samples collected into aluminized breath-bag (Teobag, 1.5 L, Tessaraux Container, Burstadt, Germany) were directly connected to isotope-selective non-dispersive infrared spectrometer (IRIS, Wagner Analysen Technik, Worpswede, Germany) which allowed continuous flow of at least 500 cc of air. The infrared analysator was interfaced to a computer system. The final results of $^{13}CO_2/^{12}CO_2$ ratios measured by the isotope-selective non-dispersive infrared-spectroscopy (NDIRS) were expressed as δ $^{13}CO_2$ (per mil) values relative to the PeeDeeBelemnite standard (9). A change of the mean δ ^{13}C value over baseline (DOB) for two time points after urea capsule ingestion, of more than 5 °/$_{oo}$ was considered as positive result, as it was established and recommended previously (10) with the use of conventional isotope-ratio mass-spectrometry (IRMS). 38 mg ^{13}C-urea was found in pilot measurements (data not showed) as the dose after which breath samples collected from all HP-positive subjects (found by gold standard method and by ^{14}C-UBT) fulfilled such criterion.

Tissue-based "golden standard" methods

The HP-status of each subject was also evaluated by a gold standard of histology (any curved bacteria in antral or corpus biopsy sample was recognized as HP-positive) and rapid urease test CLOtest (Campylobacter-like Organism test, Delta West Pty Ltd, Bentlley, Western Australia Ltd). Briefly, during the gastroduodenoscopy two antral biopsy samples were taken from the antral mucosa for histology and one for rapid urease CLO-test. Histology samples were fixed in formalin, embedded in wax, sectioned in routine fashion and stained with Giemsa. Histological evaluation was performed by two independent pathologists who were blinded to the results of UBTs. Presence of curved or spiral organisms in 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). CLO-test result was assigned as HP-positive when a change of color from yellow to red was immediate or 3 h after placing the biopsy sample on plastic slides with urea and phenol red. When CLOtest remained yellow for 24 h and histology was negative the HP status was considered as negative. By adding histology grade (0—3) to the result of CLOtest (0 for negative, 1 for positive), a total score

ranging from 0 to 4 was obtained for each subject tested. The relationship between the amount of the radioactivity (in DPM) in the exhaled air (15 minutes after ^{14}C-urea capsule ingestion) and the combined score of CLOtest (0-1) plus histology (0-3) was evaluated. Similar relation with the use of ^{13}C-UBT results (expressed as δ $^{13}CO_2$ per mil) was also established.

For each patient results of the ^{13}C-urea breath test (^{13}C-UBT), ^{14}C-urea breath test (^{14}C-UBT), CLOtest, histology and results obtained for tissue-based gold standards methods (CLO test and/or histology findings) were compared.

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

RESULTS

The most often endoscopic findings among symptomatic patients enrolled for this study were duodenal ulcer (44 %), gastric ulcer (16%), gastric mucosal erosions (26%), and healthy normal subjects (14%). HP was detected by tissue-based „gold standard" (CLO-test or/and histology) in 92 (81%) out of the 114 enrolled patients. For all HP-positive patients diagnosed with ^{13}C-UBT the values of δ $^{13}CO_2$ were in excess of cut-off point (DOB > 5) at least for two collection time points 10 and/or 15 and 20 after ingestion of ^{13}C-urea containing capsule. In the 22 patients without HP defined by the gold standard, ^{13}C-UBT had a mean exhaled excess δ $^{13}CO_2$ of 1.4 per mil (SD $\pm$0.7). In 92 patients HP positively classified by gold standard (positive CLOtest and/or positive histology) ^{13}C-UBT correctly identified 89 patients (sensitivity 97%, specificity 92%). For those subjects the mean excess δ $^{13}CO_2$ in the expired breath collected at 15 and 20 min was 12.3 per mil and 12.2 per mil, respectively (range 4.6-31.5 per mil and 3.8-38 per mil). Typical $^{13}CO_2$ and $^{14}CO_2$ exhalation curves in studied HP-positive (with cut off values: DOB > 5 and DPM > 150) and in HP-negative (DOB < 5, DPM < 100) subjects are drawn on *Fig. 1*. In HP-positive patients $^{14}CO_2$ excretion peak occurred close to 10 min (approx. at 12 min) after urea encapsulated dose whereas the average maximal $^{13}CO_2$ output was delayed of about 2—3 min shifted more distinctly towards 15 min (maximum at approx. 14 min). In HP-negative subjects corresponding exhalation curves could not be displayed adequately on the same scale. There were no side effects from the tests and no apparent complications from the study biopsies.

Fig. 2 shows the prevalence of HP infection determined by each diagnostic methods. CLOtest was positive in 70 (61%) and histology positive in 79 (69%) patients. Each of this tissue-based technique underestimated the true prevalence of infection as determined by the gold standard which identified HP in 92 patients (81%). Both ^{13}C-UBT and ^{14}C-UBT identified HP in 89 patient (78%). There were 25 patients with negative ^{13}C-UBT and ^{14}C-UBT results. However, one of them was positive by gold standard (only by histology) but had negative CLOtest what may be explained by the inhibition of urease

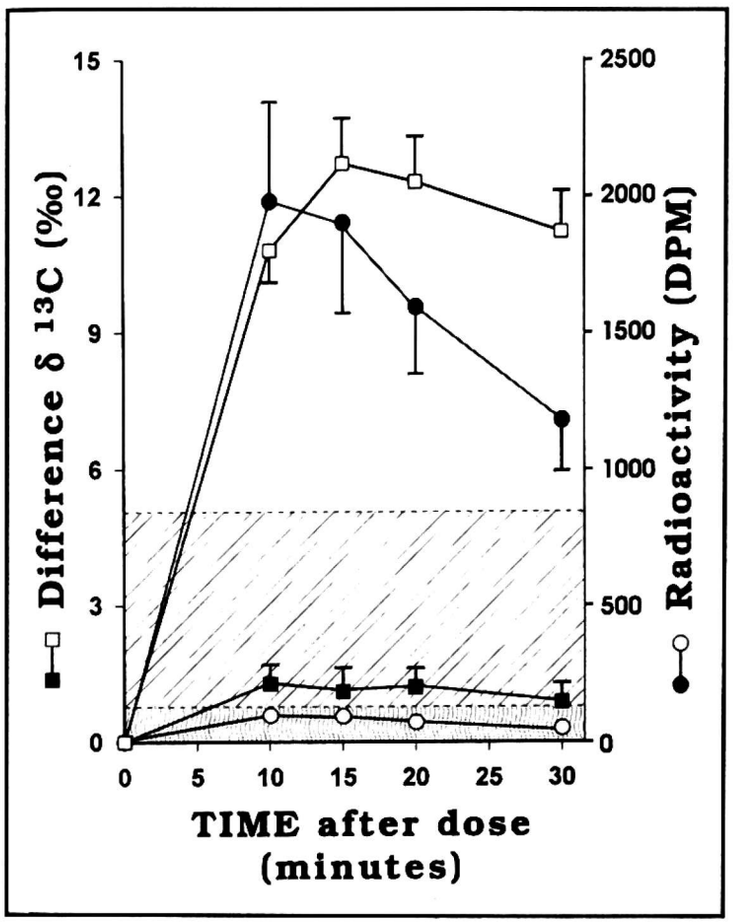

Fig. 1. Typical $^{13}CO_2$ and $^{14}CO_2$ exhalation curves in HP-positive and HP-negative subjects. Data are given as ^{13}C change (‰) over basal values for ^{13}C-UBT (squares) and as DPM (desintegrations per minute) for ^{14}C-UBT (circles). Open squares and black circles represent HP-positive subjects, open circles and black squares are HP-negative patients.

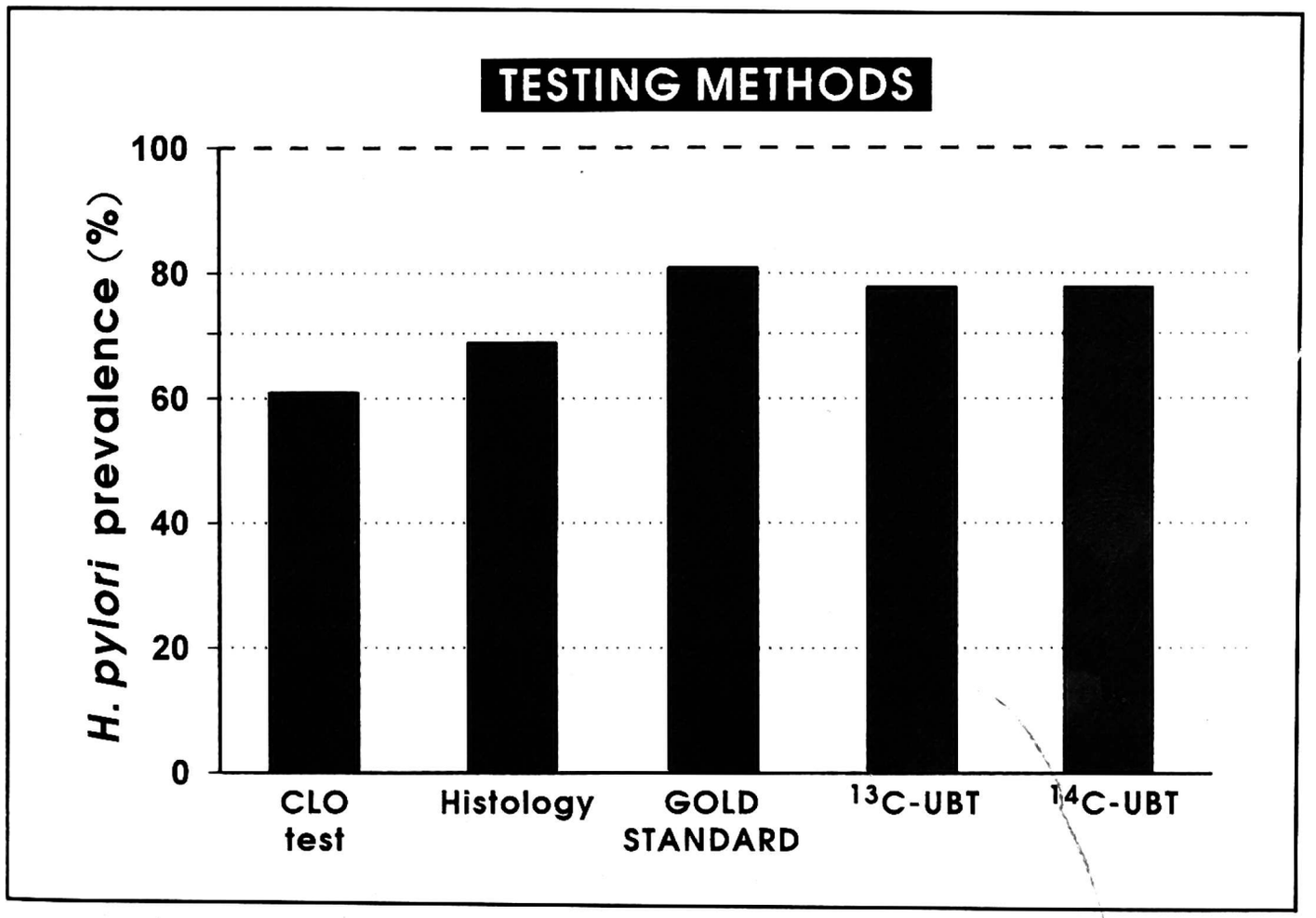

Fig. 2. HP determined by various testing methods. For 114 patients who underwent endoscopy antral specimens were evaluated by CLOtest and histology (Giemsa stain). A positive by the gold standard is defined as a positive CLOtest or histology. Each patient also underwent ^{13}C-UBT and ^{14}C-UBT.

activity caused by undefined medication taken by the patient prior to the tests what he reported afterward. For all 89 patients ^{14}C-UBT was in full concordance with ^{13}C-UBT and radioactivity measured in breath samples collected at 15 and 20 min after ingestion of capsulated ^{14}C-urea was above the cut-off value (>150 DPM). None of the breath samples collected for HP-negative patient exceeded 100 DPM.

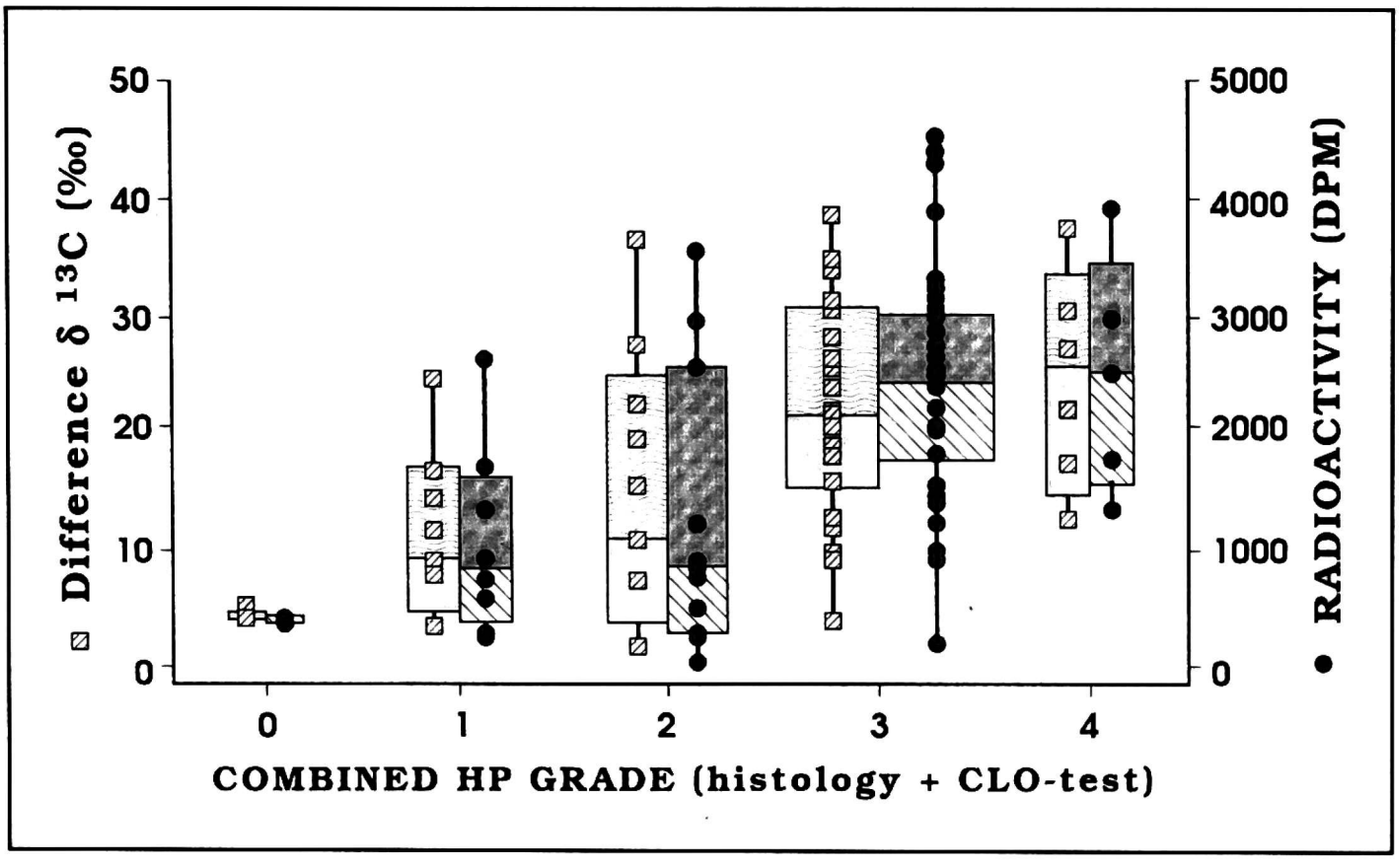

Fig. 3. Means, upper and lower quartiles for HP grades 1—4. Note: Boxplots show means, upper and lower quartiles; squares for ^{13}C-UBT and circles for ^{14}C-UBT show location of individuals. Only HP-positive patients are shown. HP grade was defined by summing histology and CLOtest results from initial histological review. Thus, zero grade was a patient in whom both tests (histology and CLOtest) 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). The relations were highly significant: $p < 0.001$, R-sq. +0.286 for ^{13}C-UBT and R-sq. +0.284 for ^{14}C-UBT.

The results of ^{13}C-UBT (and ^{14}C-UBT) and combined results constructed by adding histology grade (0—3) to the result of the CLOtest (0—1) in the same subjects highly correlated giving a total scores ranging from 0 to 4 *(Fig. 3)*. In this manner only HP-positive patients are considered since nearly all HP-negative patients gave histology grades and $^{13}CO_2$ per mil values or DPM results near zero. There was no or very little overlap between grade 1—2 and grade 3—4. Both breath tests tended to be more strongly positive in patients with massive mucosal colonization with HP, grades 3—4 (histology grade 2 or 3 and CLOtest 1). For grades 1—2 HP colonization of HP was found to be patchy or sparse. The relations were highly significant ($p < 0.001$, R-sq. +0.286 for ^{13}C-UBT and R-sq. +0.284 for ^{14}C-UBT).

DISCUSSION

Current gold standard for diagnosis of HP gastric mucosal infection requires endoscopic antral biopsy for rapid urease test (e.g. CLOtest) and/or histology. We compared the golden standard to a new low-dose capsule-based 38 mg ^{13}C-urea test (^{13}C-UBT) and mini-dose capsule-based 37 kBq ^{14}C-urea test (^{14}C-UBT) in 114 consecutive patients undergoing upper endoscopy. We reported previously that the use of ^{14}C-urea of low activity (37 kBq) enclosed in easy dissolving capsule is highly sensitive and accurate test for the diagnosis of HP gastric infection (7). In this study, we used ^{13}C-urea also in a solid state with far lower dose of ^{13}C-urea than it was described elsewhere (6). By adding histology grade to the results of CLO-test we confirmed positive correlation between radioactivity obtained for ^{14}C-UBT, δ $^{13}CO_2$ values ($^{13}CO_2/^{12}CO_2$ ratios) at 15 minutes and combined histology + CLO-test scores. Since, the ^{13}C-urea is not exposed to mouth bacterial urease and possible oral contamination is eliminated, variation in HP-negative subjects (near baseline) is much less and improved discrimination between HP-positive and HP-negative persons is achieved. The separation of HP-negative and HP-positive is far greater than with liquid based (urea solution) tests. Therefore, the test can be performed using an isotope at much lower dose i.e. only 38 mg of ^{13}C-urea in contrast with 75, 100 or 150 mg ^{13}C-urea proposed so far in liquid form in various ^{13}C-UBT modifications (10, 11). Various urea test protocols recommend additional stages and precautions, such as the use of the mouth washing with citric acid solution to decrease buccal urease activity, preliminary meal to delay gastric emptying, and/or the addition of a „cold" urea substrate to saturate the urea enzyme system (12—14). Our previous results obtained with ^{14}C-urea confined exclusively to the mouth (7) showed that these precaution can be avoided without any loss of diagnostic accuracy in a case when capsule-based ^{13}C-urea is used. Additional advantage of such modification is shorter time during which breath sample may be collected (10 or 15 min after urea ingestion) without any risk of the contact of tested urea with oral, or/and oropharynx urease.

Shorter time (after urea ingestion) of breath sample collection may also be important for diagnostic value especially for persons with fast or even rapid gastric emptying such as occurs in some patients with gastric motor disorder. Swallowed amount of ^{13}C-urea in solid state (38 mg) is about half dose of what was proposed as the European standardized protocol for ^{13}C-UBT in which ^{13}C-urea was given in liquid form orally (15). Since much lower amount of ^{13}C-urea is needed in our modification, the method of capsule-based ^{13}C-UBT becomes also significantly cheaper. As the instrumentation used here (NDIRS — non dispersive infrared spectrometer) does not require an experienced operator the final analysis of breath samples is quicker. Final result can be

obtained at site laboratory within an half an hour, thus, allowing physician decisions to be made at the same day or even at the same hour of clinic visit.

To our knowledge this is the first published studies in which capsule-based modification of ^{13}C-UBT with the use of only 38 mg of ^{13}C-urea is described. Finnally, we conclude that capsule-based low-dose ^{13}C-UBT is an excellent, low cost, non-invasive test giving identical result to that obtained with mini-dose radioactive ^{14}C-UBT and that ^{13}C-UBT should be recommended particularly in cases when the use of radioactive urea is contraindicated.

Acknowledgments: The author would like to thank for financial support to Mr. E. Surowka, B.Sc., Director of POLFA Pharmaceutical Company, Krakow, Poland. Our thanks go also to Dr. D. Kulig, Head of Research Laboratory, Polfa, Krakow, Poland, for his valuable technical advice. We are deeply obliged for excellent editorial help to Ms. Kinga Ogonowska.

This low-dose ^{13}C-urea capsule preparation for breath test in detecting of *Heliocobacter pylori* infection has been filed as a patent application with the Patent Office.

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Received: July 25, 1996
Accepted: August 2, 1996

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