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Original Article

Validation of Octanoate Breath Test for Measuring Gastric Emptying in Rats

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Background/Aims
Lack of simple and repeatable tests hampers gastric emptying studies in rats. The aim of this study was to adapt the ^{14}C-octanoate solid gastric emptying breath test for application in rats, and to validate it against radioscintigraphic method.

Methods
After ingestion of a meal containing 3 mCi ^{99m}Tc and 2 μCi ^{14}C-octanoate, 23 male Wistar rats were placed on a gamma camera in a airflow container. Scintigraphic images were taken at regular intervals. The amount of $^{14}CO_2$ in a regularly replaced hyamine hydroxide solution, capturing CO_2 in the outflow air, was counted using liquid scintillation spectrometry. ^{99m}Tc gastric retention curves and $^{14}CO_2$-excretion curves were fitted to their respective data. Three rats underwent the same procedures after administration of atropine.

Results
Overall $T_{r10\%}$ (time at which 10% of the original amount of ^{99m}Tc remained in the stomach) was 355 ± 64 minutes; $T_{e90\%}$ (time at which 90% of total amount of $^{14}CO_2$ was excreted) was 325 ± 106 minutes. Their correlation coefficient was 0.71, R-square 0.50 and $P < 0.005$. $T_{r1/2}$ (50% of original amount of ^{99m}Tc remained) was 124 ± 28 minutes; $T_{e1/2}$ (50% of total amount of $^{14}CO_2$ excreted) 114 ± 32 minutes. Their correlation coefficient was 0.83 with R-square of 0.69 and $P < 0.00005$. In 12 immobilized animals correlation was even better: correlation coefficient 0.84; R-square 0.71 and $P < 0.001$ ($T_{r10\%}$ was 388 ± 117 minutes; $T_{e90\%}$ 532 ± 219 minutes; $T_{r1/2}$ of 165 ± 54 minutes; $T_{e1/2}$ of 175 ± 67 minutes). Atropine significantly lengthened all emptying times: 904 ± 307 and 1461 ± 684 minutes for $T_{r10\%}$ and $T_{e90\%}$, respectively; and 432 ± 117 minutes for $T_{r1/2}$ and 473 ± 190 minutes for $T_{e1/2}$.

Conclusions
We adapted and validated the ^{14}C-octanoate gastric emptying breath test for application in rats.
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Key Words
Breath tests; ^{14}C-octanoate; Gastric emptying; Rats

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Introduction

Gastric emptying is a tightly regulated process, which is influenced by a number of physiological (e.g., satiety and stress) and pathophysiological (e.g., autonomous neuropathy, surgery and drugs) processes. Gastric emptying studies are needed for better understanding of these interactions, and are also an important tool in the development of drugs for several gastrointestinal disorders. Small animals like rats or mice are frequently used in pathophysiological and pharmacological studies, but studies of gastric motility are hampered by the lack of an easily applicable and repeatable gastric emptying test for these models.

Numerous approaches have been reported for evaluating gastric emptying in rats. The most frequently used procedure consists of evaluation of marker contents in various gastrointestinal sites after sacrificing the animal.[1-4] This technique is labor-intensive and requires a large number of animals for each scheduled sampling time point, to assess the temporal evolution of gastric emptying. Other studies used serial or double sampling procedures in which gastric volume was estimated by aspiration of gastric contents.[5-7] Depending on the technique used, repeated measurements in the same animal are possible both during a single experimental session and over several experimental sessions. However, this approach has several disadvantages. First, the technique can only measure liquid gastric contents. Second, repeated handling of the animal is required for instillation and recovery of the test meal through oral gavage or via an indwelling gastric canula, and handling is known to increase plasma cortisol and catecholamines,[8] making stress a potentially relevant confounding factor. Third, surgery is required to install an indwelling gastric canula in some of these techniques. Houghton et al[9] demonstrated that even minor abdominal surgery caused delayed gastric emptying for up to 2 months after laparotomy. Scintigraphic methods, the gold standard in humans, are also reported in rats.[9-11] These techniques involve the use of expensive equipment and ingestion of radioactive isotopes. They also require immobilization of the rat, either by mechanical or chemical restraints, both of which can influence gastric emptying. Immobilization is necessary to maintain a constant distance of the marker-meal to the gamma camera, in order to avoid misinterpreting changes in attenuation of the gamma emissions as changes in retention of radioactivity.[12]

The present study was performed to adapt the ^{14}C-octanoate solid gastric emptying breath test,[13,14] for application in rats and to validate it against a radioscintigraphic method. The advantages of a breath test over radioscintigraphy consist in the use of less expensive equipment and in the less strict immobilization requirements. Similar to scintigraphy, the test meal can be solid and mimic the composition of a normal diet; the test is also repeatable. The breath test takes advantage of the characteristics of octanoic acid, an 8-chain fatty acid.[15-18] This molecule is passively and rapidly absorbed by the intestinal but not the gastric mucosa, transported without prior esterification via the portal vein to the liver and oxidized to $^{14}CO_2$. The rate at which $^{14}CO_2$ is expired, is therefore proportional to the rate of arrival in the intestine.

Materials and Methods

Test Meal

The test meal, which was freshly prepared on the day of the test, consisted of 0.5 g scrambled egg yolk. This was doped with 3 mCi ^{99m}Tc-albumin colloid (Ultra Technicow, Malinkrodt Medical, Petten, The Netherlands), 2 μCi ^{14}C-octanoic acid, and sodium salt (DuPont, NEN Research, Boston, MA, USA) and then baked. Subsequently, the baked yolk was mixed with 9.5 g standard rat chow (4352 Muracon G, Nutreco Belgium NV, Gent, Belgium). Tap water (1:1) was added to form a homogenous paste (fat 6.26%, carbohydrates 33.73%, proteins 20.84% and 1.37 kcal/g). The paste was divided in aliquots of 0.5 g. From previous (unpublished) observations we knew that this amount was rapidly ingested by most animals after an overnight fast.

Animals

Twenty-six male Wistar rats (250-400 g) were housed in wire-mesh bottom cages in a restricted access room with a 12-hour light-dark cycle. Standard laboratory rat chow was available ad libitum until the night before the test, when it was removed. Tap water was freely available at all times, except during the test.

Test Procedures

Protocol 1: Standard breath test emptying protocol

After an overnight fast, 10 rats spontaneously ate the 0.5 g test meal, while 4 rats were given 0.5 g of the test meal by gavage. Immediately after ingestion of the test meal, the rat was placed in an airtight container through which a continuous airflow (80% N_2 and 20% O_2) of 0.5 mL/min was maintained (Fig. 1). The dimensions of the container (diameter 6 cm and length 23 cm) were

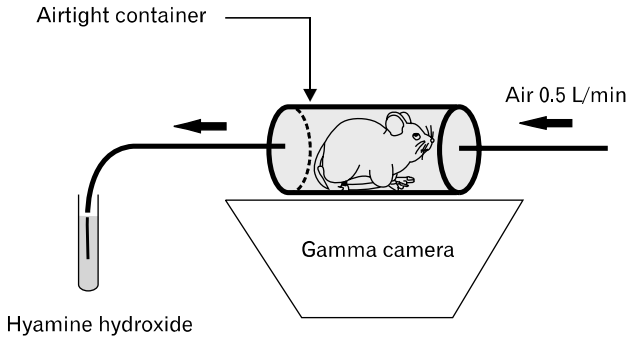

Figure 1. Set-up of the experiment. After food intake, the rat is placed in an airtight container through which a continuous airflow of 0.5 L/min is maintained with outgoing air bubbles through a tube containing the CO_2-trapper hyamine hydroxide. The airtight container is placed on a gamma camera.

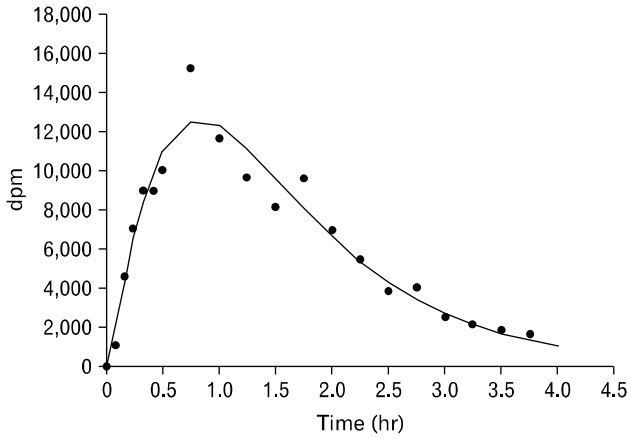

Figure 2. Examples of scintigrafic and breath test curves.

chosen to allow forward and backward movements of the rat whilst keeping the stomach-camera distance relatively constant. The container was placed on a single head gamma camera (Pho/Gamma IV, Searle, Nuclear Chicago Division, Des Plaines, IL, USA). The gas outflow of the container was bubbled through a tube filled with a solution containing 2 mmol hyamine hydroxide, a CO_2-trapper, and thymolphthaleine as indicator. Decoloration of the fluid indicated saturation with CO_2; this never occurred with a sampling time of 5 minutes, therefore, all the CO_2 expired during this time was captured. Preliminary tests with 2 tubes in series showed that more than 95 percent of CO_2 was retained in the first tube; hence only 1 tube was used in the present study. A hyamine hydroxide tube was connected to the container for a period of 5 minutes before the meal, at every 5 minutes during the first half hour and every 15 minutes for the remainder of the test period. After the initial image immediately after ingestion of the test meal, images were obtained at every 5 minutes for the first hour, then every 10 minutes for the following hours depending on the propagation of the meal by visual inspection. The test was ended when no discernible region of interest remained in the gastric area. By this time, $^{14}CO_2$-excretion was tailing off with bulk of it being already past (Fig. 2).

Protocol 2: Immobilization protocol

To minimize variability in the scintigraphic data due to incomplete immobilization, a second series of tests were performed. After an overnight fast, 8 rats spontaneously ate the 0.5 g test meal at the same session and 1 rat was given 0.5 g of the test meal by gavage. Immediately after ingestion of the test meal the rat was placed in a restraining device, which was inserted into the airtight container, while taking care to keep the position of each rat identical in respect to the gamma camera. After an initial image immediately after ingestion of the test meal, images were obtained at every 5 minutes for the first hour, then at every 15 minutes for the remainder of the test period. Once before the test meal and then every 5 minutes during the first half hour and every 15 minutes for the remainder of the test period, the hyamine hydroxide vial was connected to the container for a period of 5 minutes. Again, the test was ended when there was no longer any discernible region of interest in the gastric area.

Protocol 3: Pharmacological intervention

To investigate the effect of inhibition of cholinergic blockade, 3 rats were given atropine sulphate (Sigma Chemical Company, St. Louis, MO, USA) of 5 mg/kg intraperitoneally after an overnight fast. Twenty minutes afterwards they spontaneously ate the 0.5 g test meal. The rest of the procedure was identical to test 2, except that it was terminated 7 hours after ingestion of the test meal. All procedures were approved by the Animal Care and Use Committee of the university.

Analysis of the ^{99m}Tc-albumin Colloid Gastric Retention Curve

The region of interest was drawn for every image and its amount of radioactivity was measured (Paragon^{TM}, Medasys, Ann Arbor, Michigan, USA). ^{99m}Tc gastric retention curves were fitted to the data, using the following model: $y = 1-(1-e^{-\alpha t})^{\beta}$. The y is the fractional dose of radioactivity retained in the stomach at time; t is the time in minutes; α and β are regression estimated constants, with α the gastric emptying rate per minute.[19,20] From the best fit the following parameters were calculated: $T_{r1/2}$ (time at which 50% of the original amount of ^{99m}Tc remained in the stomach) as $(-1/k)\ \ln(1-0.5^{1/\beta})$; and $T_{r10\%}$ (time at which 10% of the original

amount of ^{99m}Tc remained in the stomach) as (-1/k) ln(1-0.9$^{1/\beta}$).

Analysis of the ^{14}C-octanoate Gastric Emptying Breath Test

After adding 10 mL of scintillation solution (Hionic Fluor, Packard Instrument Company, Dowers Grove, IL, USA), the amount of $^{14}CO_2$ in the solution was counted using a liquid scintillation spectrometer system (model 2450 Packard; Packard Instrument Company). $^{14}CO_2$ excretion curves were fitted to the data, using models given by the following formulas: $y = mk\beta e^{-kt}(1-e^{-kt})^{\beta-1}$ or $y = at^b e^{-ct}$.[13,14] The y is the $^{14}CO_2$ excretion in breath per hour; t is the time in hours; m, k, β, a, b and c are regression estimated constants, with m the total amount of $^{14}CO_2$ recovered when time is infinite. From the best fit the following parameters were calculated: $T_{e1/2}$ (time at which 50% of the total amount of $^{14}CO_2$ was excreted) as (-1/k) ln(1-0.5$^{1/\beta}$) × 60; $T_{e90\%}$ (time at which 90% of the total amount of $^{14}CO_2$ was excreted) as (-1/k) ln(1-0.91$^{1/\beta}$) × 60; and gastric emptying coefficient (GEC, global index for the gastric emptying rate) as ln(a). The lag phase has been defined in different ways over time: originally it represented the initial delay in gastric emptying of solids as compared to liquids. Later, its value was determined as the point of inflection of the cumulative excretion curve, corresponding to the time when the peak of the excretion curve was reached or even as the time at which 10% of the total amount of $^{14}CO_2$ was excreted.[13,14,19-22]

Statistical Methods

$T_{r10\%}$, $T_{e90\%}$, $T_{r1/2}$, $T_{e1/2}$ and GEC were calculated (mean ± SEM). Regression analysis (correlation coefficient, R-square and *P*-value) between $T_{r10\%}$ and $T_{e90\%}$ was performed on the pooled data of all 26 rats and on the immobilized and free group separately. According to the method proposed by Bland and Altman[23,24] for determination of the limits of agreement, 2 plots for visual analysis were made: the breath test results against the scintigraphic ones and the difference in measurements against the average of the 2 measurements. The same procedures were followed for $T_{r1/2}$ and $T_{e1/2}$. Regression analysis was also performed between $T_{r1/2}$ and GEC; but no limits of agreement could be determined since the units of measurement were different (minutes versus a mathematical parameter). To study the effects of immobilization and gavage we performed *t* tests of all emptying parameters between spontaneously fed free and immobilized rats and between spontaneous and gavage fed animals. We also performed *t* tests between atropine pretreated and not pretreated immobilized animals.

Results

Pooled Data Scintigraphic $T_{r10\%}$ and Breath Test $T_{e90\%}$

For all 26 rats, the scintigraphic $T_{r10\%}$ was 355 ± 64 minutes; $T_{e90\%}$ for the breath test was 325 ± 106 minutes. Their correlation

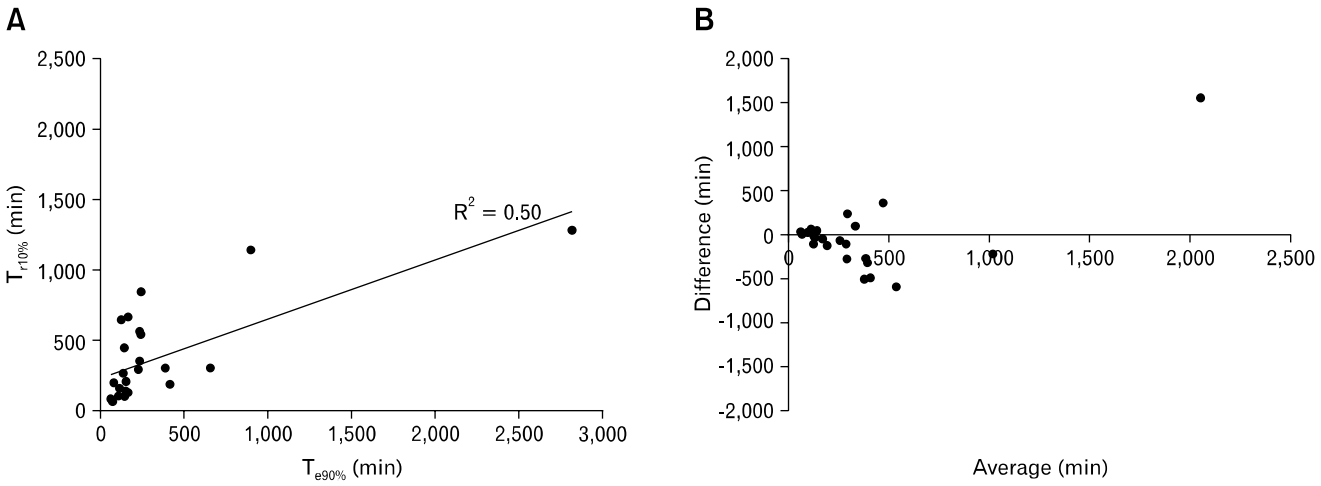

Figure 3. The left panel (A) depicts correlation $T_{e90\%}$ (time at which 90% of the total amount of $^{14}CO_2$ was excreted) and $T_{r10\%}$ (time at which 10% of the original amount of ^{99m}Tc remained in the stomach). The right panel (B) plots the difference in measurements against the average of 2 measurements ($T_{e90\%}$ and $T_{r10\%}$).

coefficient was 0.71; R-square 0.50 and $P < 0.0005$ (Fig. 3). We also plotted the difference in measurements against the average of the 2 measurements, according to the method proposed by Bland and Altman (Fig. 3).[23,24] This figure shows the scatter of differences increasing as the average increases. As a consequence of this, the limits of agreement would be wider apart than necessary for the smaller values and narrower than they should be for larger values; we did not perform a formal analysis on the limits of agreement. In the 14 free moving rats from the first test, the scintigraphic $T_{r10\%}$ was 328 ± 68 minutes; $T_{e90\%}$ for the breath test was 147 ± 18 minutes. Their correlation coefficient was 0.59; R-square 0.35 and $P < 0.05$. The 12 immobilized rats from test 2 had a scintigraphic $T_{r10\%}$ of 388 ± 117 minutes; $T_{e90\%}$ for the breath test was 532 ± 219 minutes. Their correlation coefficient was 0.84; R-square 0.71 and $P < 0.001$. The large variability in these rats was caused by much higher values of emptying times after atropine (cfr. infra): 904 ± 307 versus 216 ± 49 minutes and 1461 ± 684 versus 223 ± 35 minutes for $T_{r10\%}$ and $T_{e90\%}$, respectively.

Pooled Data of Scintigraphic $T_{r1/2}$ and Breath Test $T_{e1/2}$

For all 26 rats, the scintigraphic $T_{r1/2}$ was 124 ± 28 minutes; $T_{e1/2}$ for the breath test was 114 ± 32 minutes. Their correlation coefficient was 0.83; R-square 0.69 and $P < 0.00005$ (Fig. 4). Again, the plot of difference in measurements against the average of 2 measurements showed an increased scatter of differences with increasing averages: no formal analysis of the limits of agreement was attempted (Fig. 4). In the 14 free rats from the first test, the scintigraphic $T_{r1/2}$ was 89 ± 19 minutes; $T_{e1/2}$ for the breath test was 61 ± 8 minutes. Their correlation coefficient was 0.79; R-square 0.62 and $P < 0.001$. The 12 immobilized rats from test 2 had a scintigraphic $T_{r1/2}$ of 165 ± 54 minutes; $T_{e1/2}$ for the breath test was 175 ± 67 minutes. Their correlation coefficient was 0.84; R-square 0.71 and $P < 0.001$. Again, the large variability was caused by the much higher values after atropine (cfr. infra): 432 ± 117 versus 76 ± 17 minutes for $T_{r1/2}$ and 473 ± 190 versus 76 ± 14 minutes for $T_{e1/2}$, respectively.

Scintigraphic $T_{r1/2}$ and Breath Test Gastric Emptying Coefficient

GEC in immobilized rats was 7.23 ± 0.28 (Fig. 5). It showed a comparable, though inverse, correlation to the scintigraphic $T_{r1/2}$ as $T_{e1/2}$ (correlation coefficient of -0.83, R-square of 0.69 and $P < 0.001$). No correlation could be shown in the pooled data or in the free rats.

Effect of Immobilization

Parameters from 9 spontaneously fed and free-moving animals were compared by unpaired t-test with parameters from 8 spontaneously fed rats, immobilized in a restraining device. Although values tended to be higher in immobilized animals, significance was only reached with the breath test $T_{e90\%}$ (Table 1).

Effect of Gavage

Parameters from 17 spontaneously and 5 gavage fed rats

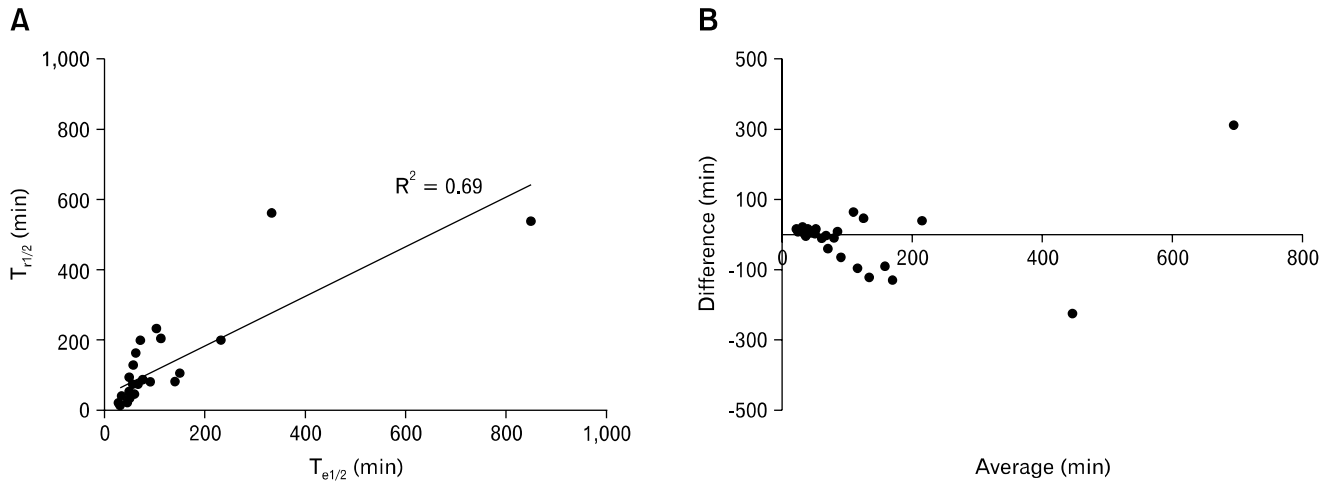

Figure 4. The left panel depicts the correlation between $T_{e1/2}$ (time at which 50% of the total amount of $^{14}CO_2$ was excreted) and $T_{r1/2}$ (time at which 50% of the original amount of ^{99m}Tc remained in the stomach). The right pannel plots the difference in the measurements of $T_{e1/2}$ and $T_{r1/2}$ against the average of the 2 measurements.

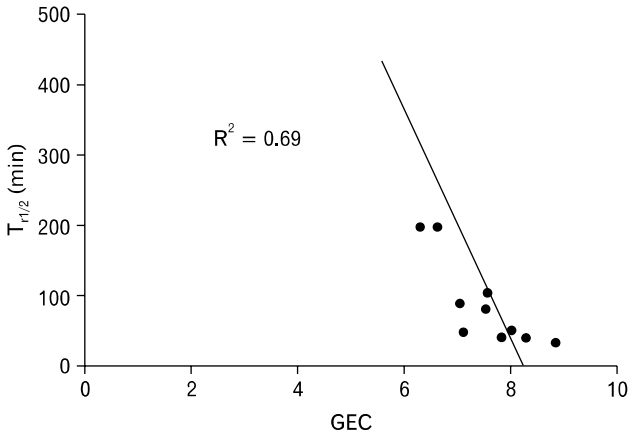

Figure 5. Correlation gastric emptying coefficient and $T_{r1/2}$ (time at which 50% of the original amount of ^{99m}Tc remained in the stomach) in immobilized rats. GEC, gastric emptying coefficient.

Table 1. Free Versus Immobilized Animals

	Free animals	Immobilized animals	*P*-value
Scintigrafic data (min)			
$T_{r1/2}$	48 ± 9	74 ± 20	NS
$T_{r10\%}$	204 ± 60	200 ± 52	NS
Breath test data (min)			
$T_{e1/2}$	47 ± 6	76 ± 15	NS
$T_{e90\%}$	119 ± 16	221 ± 40	< 0.05

$T_{r1/2}$, time at which 50% of the original amount of ^{99m}Tc remained in the stomach; $T_{r10\%}$, time at which 10% of the original amount of ^{99m}Tc remained in the stomach; $T_{e1/2}$, time at which 50% of the total amount of $^{14}CO_2$ was excreted; $T_{e90\%}$, time at which 90% of the total amount of $^{14}CO_2$ was excreted.

were compared by unpaired *t* tests. Values were higher after gavage, although significance was only reached in the scintigraphic data (Table 2). Including only data from free-moving rats did not alter the significance levels.

Effect of Atropine

Unpaired *t* tests were performed on 11 spontaneously fed immobilized animals, 3 of whom pretreated with atropine. There was a significant and marked lengthening of all emptying parameters (Table 3).

Discussion

In this study we adapted the ^{14}C-octanoate breath test for use in rats. When compared to the human test, one of the major differences is the fact that in each sample, we captured all CO_2 produced during the sampling period. This means that during the first half hour of the test, when the rapid rise to the peak excretion occurs, all excreted $^{14}CO_2$ is captured (sampling during 5 minutes, with a new tube for every 5 minutes). Also, we did not express the $^{14}CO_2$ excretion as a percentage of either the administered or the total recovered amount, but as actual amount captured during the sampling period. Furthermore, since we used ^{14}C rather than ^{13}C as a marker, we were certain that all excreted $^{14}CO_2$ originated from the administered test meal itself.

The ^{14}C-octanoate breath test can be used to measure gastric emptying because the postgastric processes of intestinal absorption, transport and metabolism are thought to be constant. In humans, inter-individual but not intra-individual variations were demonstrated in healthy volunteers.[20,21] However, these volunteers included both males and females and the age difference was as much as 51 years between the oldest and youngest volunteer. In our study, as indeed in most animal studies, same sex (usually male) and similar weight animals from the same genetic pool were used. These animals had all been living under the same condi-

Table 2. Spontaneously Versus Gavage Fed Animals

	Spontaneously fed animals	Gavage fed animals	*P*-value
Scintigrafic data (min)			
$T_{r1/2}$	60 ± 11	132 ± 25	< 0.01
$T_{r10\%}$	202 ± 39	450 ± 72	< 0.01
Breath test data (min)			
$T_{e1/2}$	61 ± 8	76 ± 9	NS
$T_{e90\%}$	167 ± 24	182 ± 22	NS

$T_{r1/2}$, time at which 50% of the original amount of ^{99m}Tc remained in the stomach; $T_{r10\%}$, time at which 10% of the original amount of ^{99m}Tc remained in the stomach; $T_{e1/2}$, time at which 50% of the total amount of $^{14}CO_2$ was excreted; $T_{e90\%}$, time at which 90% of the total amount of $^{14}CO_2$ was excreted.

Table 3. With and Without Atropine

	Without atropine	After atropine	*P*-value
Scintigrafic data (min)			
$T_{r1/2}$	74 ± 20	432 ± 117	< 0.001
$T_{r10\%}$	199 ± 52	904 ± 307	< 0.01
Breath test data (min)			
$T_{e1/2}$	76 ± 15	473 ± 190	< 0.01
$T_{e90\%}$	221 ± 40	1461 ± 684	< 0.05

$T_{r1/2}$, time at which 50% of the original amount of ^{99m}Tc remained in the stomach; $T_{r10\%}$, time at which 10% of the original amount of ^{99m}Tc remained in the stomach; $T_{e1/2}$, time at which 50% of the total amount of $^{14}CO_2$ was excreted; $T_{e90\%}$, time at which 90% of the total amount of $^{14}CO_2$ was excreted.

tions. Therefore inter-individual variations of octanoate metabolism are likely to be smaller.

We found a good correlation between breath test and scintigraphic emptying parameters. As expected, this correlation was better for immobilized than for free-moving rats, probably attributable to the variations in meal-to-camera distance in free-moving rats.

The differences between results from both tests seem to increase towards the higher values. This probably reflects amplification of errors. As Choi et al[21,22] pointed out in their human studies, if the length of the collecting period is too short, the estimate of the regression parameter is less accurate, leading to overestimation of breath test, emptying times. However, even if the optimal length of testing has not been established, for each individual test, it can be postulated that the test period would be long enough if its cumulative excretion curve reached a plateau instead of still being in the ascending slope. The plateau in the cumulative excretion curve corresponds to the tailing off of the descending slope towards the horizontal axis in the excretion curve (Fig. 2). For most of the tested rats, this tailing off was present with the atropine treated rats being the exception.

Scintigraphic emptying times in our studies were as long as or longer than their breath test counterparts. This may be due to positioning of the rat: when the stomach region of interest and first part of the duodenum overlap, the initial stages of gastric emptying are invisible on the gamma camera images while $^{14}CO_2$ is already expired. Testing this hypothesis requires a dual head gamma camera, but this is not available for animal studies in our center. As a consequence, we were not able to calculate a correcting factor for the breath test emptying times, corresponding to the time needed for post-gastric emptying processing of ^{14}C-octanoic acid. Thus, it must be stressed that although there is a good correlation between scintigraphic and breath test parameters, their values are not interchangeable.

We also demonstrated that gastric emptying of solids, measured by the breath test, was much slower after administration of the anticholinergic agent atropine. The atropine dose we used was similar to previous studies,[25,26] and was half the dose reported to have a full blocking effect in studies of heart rate variability.[27] Immobilization and gavage tended to be associated with longer emptying times, although significance was never reached in the breath test, possibly due to the relatively low number of animals studied, especially in the gavage group.

In conclusion, we adapted and validated the ^{14}C-octanoate solid gastric emptying breath test for application in rats. The availability of this test offers perspectives or repeated pharmacological and pathophysiological studies of gastric emptying in rats.

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