A DISTRIBUTION STUDY WITH 14C-OTILONIUM BROMIDE IN THE RAT: EVIDENCE FOR SELECTIVE TROPISM FOR LARGE INTESTINE AFTER ORAL ADMINISTRATION

STEFANO EVANGELISTA, PASCAL COCHET, NORBERT BROMET, MARCO CRISCUOLI, AND CARLO ALBERTO MAGGI

Menarini Ricerche S.P.A., Firenze, Italy (S.E., M.C., C.A.M.) and Biotec Centre, Orleans, France (P.C., N.B.)

(Received for publication November 9, 1999; accepted March 9, 2000)

This paper is available online at http://www.dmd.org

ABSTRACT:

The aim of this study was to determine the plasma levels and the tissue distribution of otilonium bromide, measured as total radioactivity, after oral administration of 2 mg/kg of 14C-labeled drug to rats. Radioactivity levels were very low in the plasma (ranging from 2.7 ng Eq/ml at 1.5 h to 0.6 ng Eq/ml at 24 h) as compared with those found in the gastrointestinal (GI) tract, indicating negligible systemic otilonium bromide absorption. Results from both quantitative radioluminography of whole body tissue distribution and radioassay of dissected parts of the GI tract carried out with liquid scintillation counting clearly demonstrate the presence of radioactive compounds in the walls of the GI tract at all sacrifice times. In the other tissues and organs examined, radioactivity was only found in trace amounts in the liver. The presence of radioactivity in the GI walls reflected the transit kinetics of drug-enriched contents. The radioactivity in large intestine walls was measurable at otilonium bromide concentrations in the range of micromole equivalents/kg, from 4 to 8 h after drug administration. Total body radioactivity recovery was 95, 101, 24, and 9% at 1.5, 4, 8, and 24 h, respectively. In conclusion, orally administered 14C-otilonium bromide is poorly absorbed systemically, as indicated by the very low plasma radioactivity levels, but it is able to effectively penetrate into the large intestine walls, a recognized target for drugs oriented toward irritable bowel syndrome therapy.

Otilonium bromide (diethylmethyl[[(octyloxy-2 benzamido)-4 benzoyloxy][2ethyl]ammonium bromide) is a quaternary ammonium salt possessing gastrointestinal (GI) spasmolytic properties, and used world-wide for the treatment of irritable bowel syndrome (IBS). A recent meta-analysis of double-blind randomized placebo-controlled trials of various smooth muscle relaxants (Poynard et al., 1994) has ranked otilonium bromide among the leading treatments in IBS when global assessment, pain relief, and absence of side effects were taken into account.

These clinical results reinforce the hypothesis of the marked selectivity of otilonium bromide toward the colon. Previous experimental studies have shown that otilonium bromide spasmolytic action, regardless of the nature of the agonist, was preferentially exerted in intestinal muscles, rather than in vascular or respiratory smooth muscle preparations (Manzini et al., 1984). In vitro studies have indicated that the colon is more sensitive than other GI segments to the relaxing action of otilonium bromide (Maggi and Melti, 1983; Maggi et al., 1985). This drug possesses a potent antimusscarinic and calcium antagonistic effect (Maggi et al., 1983b; Gandia et al., 1996; Evangelista et al., 1998), but is free of the typical systemic side effects of this drug type (Scarpignato et al., 1980; Maggi et al., 1983a). Moreover, when orally administered at doses that produce spasmolytic effects in humans, it was devoid of both central and peripheral atropine-like side effects (Sutton et al., 1997). All these data suggest negligible systemic absorption of otilonium bromide, whereas in vitro studies performed using the 14C-labeled compound showed preferential binding to GI tract tissues (Amenta et al., 1991). This study was carried out to determine the quantitative and temporal tissue distribution of 14C-otilonium bromide in rats, after a single oral dose that is similar to the therapeutic dose currently administered in IBS treatment (Battaglia et al., 1998).

Materials and Methods

Animals and Treatments. Twenty male Sprague-Dawley rats (Janvier, Le Genest St. Isle, France), 240 to 260 g, were used. They were housed in a temperature- and humidity-controlled room and fasted 18 h before the experiment. Each animal received 2 mg/kg of a mixture of 14C-otilonium bromide (specific activity 58 mCi/mmol; Amersham Pharmacia Biotech, Courtaboeuf, France) and unlabeled compound (Spasmomen; Menarini Pharmaceuticals, Firenze, Italy) dissolved in a sterile physiological vehicle and slowly delivered into the stomach through a gastric cannula (5 ml/kg). The nominal radioactive dose was 150 µCi/kg.

Blood Sampling and Animal Euthanasia. Blood samples were drawn into heparinized pipettes from the retro-orbital sinus at 1.5, 4, 8, and 24 h after dosing. Plasma was separated by centrifugation (3000 rpm for 10 min) from an aliquot of each blood sample. At each sampling time, after blood collection, five animals were sacrificed by excess anesthesia with diethyl ether, and processed according to the different study protocols below.

Quantitative Radioluminography (QRLG). After sacrifice, the 12 animals (three at a time) were studied with the QRLG technique (Ullberg and Larsson, 1981) were prepared for cryosectioning by immersion of the suitably restrained body in a cooling medium at −70°C (eutectic mixture of heptane and solid carbon dioxide) for 45 min. Frozen rats were stored at −20°C until embedding into a carboxymethylcellulose matrix (2% in water) and cryosec-
tion of blocks. Animals were sliced using a bright cryomicrotome (Instrument Company Ltd., Huntingdon, England). Thirty sagittal sections of 30-μm thickness were collected and fixed on an adhesive support. Slices were dried at −20°C in a cryostat for 24 h. Twelve lyophilized sections were selected for evaluating the concentrations of 14C-otilonium bromide-associated radioactivity in duplicate or triplicate in every tissue/organ.

The bioimaging analyzer system (BAS 1800; Fuji, Tokyo, Japan) was used for the QRLG, using imaging plates (IPs), which accumulate and store radioactive energy (Mori and Hamaoka, 1994). To quantify radioluminograms, a calibrating scale of 11 radioactivity levels (range 0.7–1849 nCi/g) was prepared by the addition of 14C-otilonium bromide to rat whole blood. After liquid scintillation counting (LSC) of each standard, precooled blood samples containing 14C-otilonium bromide were poured into 0.7-mm-diameter pores drilled in a block of carboxymethylcellulose matrix maintained at −20°C. Sections of 30-μm thickness were obtained by cryomicrotomy and processed in the same way as the previous ones. The tapes with lyophilized material were attached to rigid supports and identified with radiolabeled ink (100 μCi/ml).

The IPs were exposed to whole body freeze-dried sections of rats together with radiolabeled blood standards. To improve resolution, close contact between histological sections and IP was ensured using X-ray cassettes. At the end of exposure (72 h), each IP was inserted into the image reading unit and scanned with a laser beam (100 μm of pixel resolution). Radioactivity was detected and quantified from 16-bit numbered images using TINA Fuji software, and results were expressed as photostimulated luminescence (PSL) units corrected for background per unit area (PSL/S, i.e., [PSL-background]/mm²). The PSL/S data were directly proportional to the amount of radioactivity that had penetrated the sample. A calibration curve (linear regression analysis of log-data were directly proportional to the amount of radioactivity that had penetrated the sample). A calibration curve (linear regression analysis of log-
data were directly proportional to the amount of radioactivity that had penetrated the sample). A calibration curve (linear regression analysis of log-

Radioassay Study in the GI Tract. For this study, eight animals were used (two animals per sacrifice time). The abdomen was carefully opened with scissors and the alimentary tract was removed from the abdominal cavity. Ten different samples were obtained from the GI tract: stomach wall, stomach contents, duodenum wall, jejunum wall, ileum wall, small intestine contents, cecum wall, cecum contents, colon plus rectum walls, and contents of colon plus rectum. The contents of stomach, small intestine, cecum, and colon plus rectum were separately expelled by manual pressure, and mixed with a large amount of distilled water in a 100-ml plastic container. All tissues were then rinsed with a small amount of water, blotted dry, weighed, and scissor-minced in a 20-ml polyethylene vial. The weighed carcasses (without the GI tract), sampled tissues, and macerated contents were stored at −20°C until used for radioactivity analysis.

Sample Processing and Radioactivity Measurements. Blood (100 mg) was digested in a scintillation vial with 1 ml of Soluene350/isopropanol mixture (1:1, v/v) at 50°C for 30 min; then 30% H₂O₂ (0.5 ml) was added before additional incubation at room temperature and at 50°C (30 min). Pico-Fluor (15 ml; Packard) was added 2 h before counting. Plasma (200 mg) was mixed with 6 ml of Pico-Fluor in a scintillation vial and counted. Weighed tissue samples were digested with 1 N NaOH (10 ml) at 60°C for 48 h; an aliquot of the solution (250 mg) was then mixed with 15 ml of Pico-Fluor in a scintillation vial 6 h before counting. GI contents, diluted and weighed, were homogenized with an Ultra-Turrax blender; an aliquot of the suspension (250 mg) was then incubated at 60°C for 48 h with 20 ml of Soluene350 (1 ml) in a scintillation vial. Pico-Fluor (15 ml) was added 6 h before counting. Each rat carcass was digested by incubating with 250 ml of 1 N NaOH at 60°C for 72 h; then 1 ml of solution was mixed with 15 ml of Pico-Fluor 12 h before counting.

Radioactivity was counted in a liquid scintillation spectrometer (1900 CA; Packard). Blank biological medium treated like other tissues and fluids gave no significant radioactivity signal.

The low quantitation limit (LQL) was set at 25 dpm/100 mg blood (ca. 0.11 nCi/g, i.e., 1.4 ng Eq otilonium bromide/g) and 20 dpm/300 mg plasma (ca. 0.03 nCi/g, i.e., 0.4 ng Eq otilonium bromide/g). For samples from the GI tract, the LQL was defined as 25 dpm/200 mg. Approximate radioactivity elimination half-lives were calculated by linear regression analysis of a semilogarithmic plot of concentration values (from the peak on) versus time.

Results

Blood and Plasma Levels. As shown in Table 1, very low levels of radioactivity were reached in plasma and blood after oral administration of 14C-otilonium bromide. In whole blood, radioactivity concentrations were comparable between 1.5 and 8 h, and declined at 24 h after administration. Plasma sample levels gradually declined from 1.5 h after administration (2.7 ± 0.6 ng Eq/ml) to values that were close to the LQL at the last sampling time (0.6 ± 0.1 ng Eq/ml).

Distribution of 14C-Otilonium Bromide in Whole Animal by Radioluminography. The digitalized images obtained from QRLG show the whole body location of 14C-otilonium bromide and any labeled metabolites. The presence of radioactivity is indicated in these radioluminograms by the blackened areas (the higher the radioactivity

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Blood</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>S.D.</td>
<td>Mean</td>
</tr>
<tr>
<td>1.5</td>
<td>5.5</td>
<td>3.4</td>
</tr>
<tr>
<td>4</td>
<td>3.2</td>
<td>0.8</td>
</tr>
<tr>
<td>8</td>
<td>5.3</td>
<td>0.3</td>
</tr>
<tr>
<td>24</td>
<td>2.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Radioactivity concentrations [Mean and S.D. (n = 5), expressed as nanogram equivalents otilonium bromide per milliliter in blood and plasma samples at various times after 2 mg/kg p.o. of 14C-otilonium bromide]
radioactivity concentrations was observed, with the exception of colon contents, where a higher value than the upper quantification limit was still found. The second highest levels were associated with the cecum contents (13.09 μg Eq/g). The small intestine contents, the cecum, and colon walls had values of 1.47, 1.10, and 1.07 μg Eq/g, respectively. The stomach mucosa (0.69 μg Eq/g) and the small intestine wall (0.20 μg Eq/g) were somewhat less radioactive. Very low radioactivity levels (<0.03 μg Eq/g) were found in the stomach wall and contents and in the liver. As expected, no radioactivity was again found in other organs.

At the last sacrifice time (24 h, Fig. 1D), radioactivity had significantly decreased in all sections of the GI tract. The colon and cecum contents were again found to be the samples that were richest in labeled compound(s) (5.56 and 2.07 μg Eq/g, respectively). However, the colon wall (0.32 μg Eq/g), as well as the stomach mucosa (0.23 μg Eq/g) and the small intestine contents (0.26 μg Eq/g), still showed marked levels. Weaker activities were found in the cecum walls (0.06 μg Eq/g) and in the small intestine wall (0.02 μg Eq/g). No radioactivity was detectable at this time in the liver and other tissues.

**Distribution of 14C-Otilonium Bromide in GI Tract by Direct Radioassay.** The temporal distribution of radioactivity in GI contents and walls, as assessed by LSC, is shown in Table 2 and Fig. 2, where data are reported as a percentage of the administered dose or as microgram equivalents of otilonium bromide per gram, respectively. At the first sampling time (1.5 h), most of the administered radioactive dose (82%) was recovered in the GI contents: 12% in the stomach, 65% in the small intestine, and 5% in the cecum. A considerable part (99%) was also associated with the GI membranes. Jejunum, ileum, and stomach walls showed the highest concentrations of labeled compound with 5.7, 11.1, and 3.4 μg Eq/g, respectively (Fig. 2). Much lower amounts were present in duodenum, colon-rectum, and cecum walls. An additional 3% of the dose was measured in the carcass, and the overall radioactivity recovery was close to 95% (Table 2).

Four hours after drug administration, about 99% of radioactivity was associated with the GI contents (3% in the stomach, 11% in the small intestine, 36% in the cecum, and 49% in the colon and rectum;

<table>
<thead>
<tr>
<th>GI Tissue or Contents</th>
<th>Sacrifice Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5 h</td>
</tr>
<tr>
<td>Stomach wall</td>
<td>1.02</td>
</tr>
<tr>
<td>Stomach contents</td>
<td>12.30</td>
</tr>
<tr>
<td>Duodenum wall</td>
<td>0.11</td>
</tr>
<tr>
<td>Jejunum wall</td>
<td>5.56</td>
</tr>
<tr>
<td>Ileum wall</td>
<td>3.64</td>
</tr>
<tr>
<td>Small intestine contents</td>
<td>65.14</td>
</tr>
<tr>
<td>Cecum wall</td>
<td>0.02</td>
</tr>
<tr>
<td>Cecum contents</td>
<td>4.76</td>
</tr>
<tr>
<td>Colon and rectum walls</td>
<td>0.01</td>
</tr>
<tr>
<td>Colon and rectum contents</td>
<td>0.11</td>
</tr>
<tr>
<td>Carcass without gastrointestinal tract</td>
<td>2.58</td>
</tr>
<tr>
<td>Total recovery</td>
<td>95.24</td>
</tr>
</tbody>
</table>
Table 2). The remainder was mainly detected in the colon-rectum and jejunum walls, where the radioactivity concentration reached 4.0 and 0.9 mg Eq/g, respectively (Fig. 2).

At 8 h after drug administration, only 24% of administered radioactivity was still present in the body (Table 2). It was mainly associated with the contents of the large intestine (22%). As shown in Fig. 2, measurable radioactivity levels were still found in the walls of the cecum (2.0 μg Eq/g) and colon-rectum (1.2 μg Eq/g).

At 24 h after drug administration, 9% of the dose was recovered in sampled tissues and contents (Table 2). The radioactivity was mainly present in the large intestine contents, but the concentration in the various intestinal wall samples was still quantifiable (0.33 and 0.30 μg Eq/g in the cecum and colon-rectum walls, respectively). Approximate apparent half-lives for the elimination of radioactive compound(s) from the GI walls were 2.5 h for the stomach, 4.0 h for the small intestine, 6.3 h for the cecum, and 6.5 h for the colon.

Discussion

The results presented above indicate that otilonium bromide has poor systemic absorption, as witnessed by the very low values of total radioactivity in blood and plasma after oral administration of the 14C-labeled compound to rats. In fact, the plasma levels found after a dose of the drug (2 mg/kg p.o.) that is close to that used in humans to treat IBS (Battaglia et al., 1998) are always at least 1000 times lower than those reached in the walls of the large intestine. Moreover, these plasma values (C_max = 2.7 ng Eq/μl) are far below the otilonium bromide concentration (0.56 μg/ml or 1 μM) needed to exert a spasmolytic activity through its combined calcium channel blocking, tachykinin NK2 receptor antagonist, and antimuscarinic activity (Maggi et al., 1983b; Gandia et al., 1996; Evangelista et al., 1998; Santicioli et al., 1999).

Two studies in humans have shown that otilonium bromide plasma levels after oral administration were undetectable (Signorini et al., 1984; Sutton et al., 1997). The plasma levels reached in this study substantiate the concept that otilonium bromide spasmolytic activity occurs through local and selective activity in the intestine. On the other hand, preliminary studies carried out with a higher dose (50 mg/kg p.o.) that is able to block stimulated GI motility in dogs (Giachetti, 1991), have shown that 14C-otilonium bromide was concentrated in the intestine wall and that the radioactivity persisted up to 24 h from administration of the drug (from 77 to 5 μg Eq/g).

In this study, the radioactivity was again found almost exclusively in the GI tract. Other organs, with the exception of liver, were not radioactive at all and no blood-brain barrier transfer was recorded. The weak radioactivity measured in the liver is in agreement with the major role of this organ in the excretion of circulating otilonium bromide; about 85% in i.v. administered drug is excreted through the bile in rats (I.D. Capel and J.W. Daniel, unpublished data, on file at Menarini Ricerche). Overall, these data are in agreement with preclinical studies (Scarpignato et al., 1980; Maggi et al., 1983a) and clinical studies (Poynard et al., 1994; Sutton et al., 1997) showing that otilonium bromide lacks the typical side effects of other antimuscarinic and calcium-blocking drugs.

Regarding tissue distribution, in accordance with the movement of the gut contents across the GI tract, otilonium bromide-related radioactivity was first observed in the stomach and the small intestine walls, from which it faded away in a relatively short time. Peak levels in the large intestine tissues were attained between 4 and 8 h from
dosing, and declined slowly, still being high at 24 h after drug administration.

It is noteworthy that the concentrations reached (and maintained for a fairly long time) in these tissues are in the range of those known to exert spasmyloytic activity in in vitro studies, i.e., 1 to 5 μM (0.6–3 μg/ml) (Maggi et al., 1983b; Gandia et al., 1996; Evangelista et al., 1998; Santicioli et al., 1999). Previous in vitro studies have shown that otilonium bromide accumulates in the inner layer of the colonic circular muscle and submucosa (Amenta et al., 1991). At this level, otilonium bromide has been shown to inhibit the contractility induced by three main receptors for excitatory transmitters, i.e., muscarinic and tachykinin NK1 and NK2 receptors (Santicioli et al., 1999), and to bind with competitive interaction calcium channels and muscarinic receptors at micromolar concentrations (Evangelista et al., 1998). This myorelaxant effect exerted by otilonium bromide at the concentrations found in the GI tract is thought to influence pain sensation and impaired visceral sensitivity, which are leading signs of IBS.

Acknowledgments. We thank Prof. A. Giachetti, Dr. A. Crea, and Dr. S. Manzini for their helpful advice and suggestions, and F. Parenti and C. Azzurrini for their secretarial assistance.

References