Effects of long-term oral administration of levothyroxine sodium on glucose dynamics in healthy adult horses

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Objective—To determine the effects of long-term oral administration of levothyroxine sodium (L-T₄) on glucose dynamics in adult euthyroid horses.

Animals—6 healthy adult mares.

Procedures—Horses received L-T₄ (48 mg/d) orally for 48 weeks. Frequently sampled IV glucose tolerance test procedures were performed on 3 occasions (24-hour intervals) before and at 16, 32, and 48 weeks during the treatment period. Data were assessed via minimal model analysis. The repeatability of measurements was evaluated.

Results—During treatment, body weight decreased significantly from the pretreatment value; mean ± SD weight was 49 ± 14 kg, 43 ± 7 kg, and 25 ± 18 kg less than the pretreatment value at weeks 16, 32, and 48, respectively. Compared with pretreatment findings, 1.8-, 2.4-, and 1.9-fold increases in mean insulin sensitivity (SI) were detected at weeks 16, 32, and 48, respectively. SI was negatively correlated with body weight (r = -0.42; P < 0.001). During treatment, glucose effectiveness increased and the acute insulin response to glucose decreased. Overall mean within-horse coefficients of variation were 5% and 29% for plasma glucose and serum insulin concentrations, respectively, and 33%, 26%, and 23% for SI, glucose effectiveness, and the acute insulin response to glucose, respectively.

Conclusions and Clinical Relevance—Long-term administration of L-T₄ was associated with weight loss and increased SI in adult euthyroid horses, although other factors may have confounded results. Levothyroxine sodium may be useful for the treatment of obesity and insulin resistance in horses, but further studies are required. (Am J Vet Res 2008;69:70–81)

In previous studies, by our group, we determined that oral administration of L-T₄ induced weight loss and significantly increased SI from pretreatment values in adult euthyroid mares. There was a median decrease of 19 kg and > 2-fold increase in SI among horses that were treated with L-T₄ at dosages ranging from 24 to 96 mg of L-T₄/d for 8 weeks. However, it was not ascertained whether those alterations would persist if L-T₄ treatment were administered for a period longer than 8 weeks. This question is relevant to the use of L-T₄ for the management of obesity and IR in horses. Insulin resistance is associated with obesity in horses, and this disturbance likely plays a role in the development of pasture-associated laminitis. Chronic IR is sometimes accompanied by enlargement of adipose tissues within the neck (so-called cresty neck), obesity, and laminitis. This collection of clinical conditions has been referred to as equine metabolic syndrome. Chronic IR is associated with obesity in horses, and this disturbance likely plays a role in the development of pasture-associated laminitis.

Insulin resistance is a risk factor for pasture-associated laminitis, which is thought to be triggered by consumption of grass that is rich in water-soluble carbohyd.
body weight and increase SI and that these responses would extend beyond the 8-week time period evaluated in our previous study.3

Materials and Methods

Animals—Six healthy mares of mixed breed and Quarter Horse body type were selected for use in the study. All horses were determined to be euthyroid on the basis of serum thyroid hormone concentrations and thyrotropin-releasing hormone response test results, which have been reported elsewhere.6 Mares were chosen as the experimental unit to reduce variability associated with differences in sex. Immature and old horses were also not selected. Mean ± SD age was 8 ± 2 years (range, 6 to 10 years) and mean body weight was 501 ± 38 kg (range, 443 to 550 kg) at 5 to 9 weeks prior to treatment (pretreatment values). Horses were housed in indoor stalls (2.75 × 3.5 m) from approximately 4 PM until 7 AM and then turned out on pasture for the remainder of the day. Each horse had access to grass hay in round bales when on pasture, and 2 flakes of mixed-grass hay (approx 4.5 kg) were provided in the stall when the horse returned at 4 PM. Each horse also received 0.5 kg of oats once daily in the morning (approx 7 AM). Water was provided ad libitum. Horses were transported to the teaching hospital on the Friday before each testing week and were housed in stalls (3.7 × 3.7 m) for 7 days. Four to 6 flakes of grass hay were fed each day during these periods. The study protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Experimental design—Each day, the 6 horses were each given 48 mg (4 teaspoons) of L-T4, mixed with 30 mL of water in their morning meal of oats during a 48-week treatment period. Horses were weighed and FSIGT test procedures were performed 5 to 9 weeks prior to beginning treatment (pretreatment) and at 16-week intervals (ie, weeks 16, 32, and 48). The delay between initial testing and the beginning of the treatment period was a consequence of an unanticipated logistical issue concerning housing. Treatment was initiated in November 2004 and testing performed after 16, 32, and 48 weeks occurred in March, June, and October, respectively. Treatment periods were staggered so that 2 horses were brought into the hospital each week; all horses were evaluated over a period of 3 weeks. During each week of testing, an IV catheter was placed on Monday and FSIGT test procedures were performed on 3 occasions at 24-hour intervals (ie, on Tuesday, Wednesday, and Thursday of that same week) to assess repeatability of results. At the completion of the treatment period, horses underwent drug withdrawal during which they were orally administered 24 mg of L-T4/d for 2 weeks, followed by 12 mg of L-T4/d for 2 weeks, and then discontinuation of treatment. Horses also underwent echocardiographic examinations and routine clinico-pathologic evaluations every 16 weeks and biopsy procedures at the beginning and end of the 48-week treatment period after FSIGT procedures were completed. Results of those assessments have been published.9

FSIGT test procedure—On the first day of each testing week, each horse was weighed and a 14-gauge polypropylene catheter was inserted into the left jugular vein. Frequently sampled IV glucose tolerance tests were performed at 24-hour intervals (subsequent days) within the same week. Each horse was provided with grass hay and water ad libitum during tests. Patency of the IV catheter was maintained by infusing 5 mL of saline (0.9% NaCl) solution containing heparin into the catheter every 6 hours. An injection cap and infusion set6 (length, 30 cm; internal diameter, 0.014 cm) were attached to the catheter. Testing was initiated at approximately 10 AM. The FSIGT test procedure that was first adapted for use in horses by Hoffman et al6 was selected. Briefly, 300 mg of glucose/kg of body weight (50% [wt/vol] dextrose solution) was infused as quickly as possible (duration of infusion, < 2.5 minutes) into each horse via the infusion line and catheter, followed by infusion of 15 mL of saline solution containing heparin. Blood samples were collected via the catheter at 0 (before dextrose infusion) and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 minutes after infusion of dextrose. At 20 minutes, regular insulin7 (30 mU/kg) was rapidly infused (duration of infusion, < 1 minute), followed by an infusion of 20 mL of saline solution containing heparin. Blood samples were subsequently collected via the catheter at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 minutes after infusion of dextrose. At each time point, 3 mL of blood was withdrawn from the infusion line and discarded. A 6-mL blood sample was then collected, followed by infusion of 5 mL of saline solution containing heparin. Half of each blood sample was transferred to tubes containing sodium heparin that were immediately cooled on ice and then refrigerated. Blood samples were centrifuged to obtain plasma within 3 hours of collection. The other portion of each blood sample was placed in glass tubes containing no anticoagulant. These samples were allowed to clot at 22°C for 1 hour and then serum was obtained via low-speed centrifugation. Plasma and serum samples were stored at –20°C until further analysis.

Plasma glucose and serum insulin concentrations—Plasma glucose concentrations were measured by use of a colorimetric assay9 on an automated discrete analyzer.7 Serum insulin concentrations were determined by use of a radioimmunoassay8 that had been validated for use in horses.10 Each sample was assayed in duplicate, and intra-assay CVs < 5% or < 10% were required for acceptance of glucose and insulin assay results, respectively.

Interpretation of FSIGT test data via minimal model analysis—Values for SI, Sg, AIRg, and DI were calculated for each FSIGT test in accordance with the minimal model11 by use of commercially available software12 and previously described methods.11,12 Disposition index was calculated by multiplication of AIRg by SI.

Statistical analysis—Plasma glucose and serum insulin concentrations before FSIGT procedures and minimal model variables were examined with a repeated-measures ANOVA in a statistical program. When significance was established, multiple comparisons were made of the differences of least squares means for weeks 16, 32, and 48 with the pretreatment mean.
value by use of the Bonferroni test. Pearson correlation coefficients were calculated to examine relationships between different minimal model variables and between these variables and body weight. Coefficient of variation values were calculated to assess the within-horse repeatability of pretest glucose and insulin concentrations and minimal model values obtained from 3 FSIGT tests performed at 24-hour intervals. Mean ± SD or mean (range) values are reported. Significance was defined as a value of P < 0.05.

### Results

Among the 6 study horses, mean ± SD pretreatment body weight was 501 ± 37 kg at 5 to 9 weeks before treatment began; weight significantly (P < 0.001) decreased during the treatment period. Mean ± SD body weights were 49 ± 14 kg, 43 ± 7 kg, and 25 ± 18 kg less than the pretreatment mean value at weeks 16, 32, and 48, respectively (Figure 1). This was equivalent to loss of 10%, 9%, and 5% body weight, respectively. When the L-T₄ dosage by weight was calculated for each horse at each time point, the mean ± SD dosage was 102 ± 8 µg/kg (range, 87 to 121 µg/kg). Plasma glucose concentrations remained unaffected, but pretest insulin concentrations decreased (P = 0.046) during the treatment period (Table 1). Mean serum insulin concentration at 16 weeks was significantly lower than the pretreatment mean value.

Minimal modeling was successfully performed with data from 71 FSIGT tests, but data from 1 pretreatment test could not be modeled. Insulin sensitivity significantly (P < 0.001) increased during the treatment period; mean SI values at weeks 16, 32, and 48 were significantly higher than the pretreatment mean value (Figure 1). Compared with the pretreatment value, 1.8-, 2.4-, and 1.9-fold increases in mean SI were detected at 16, 32, and 48 weeks, respectively; there was a negative correlation (r = −0.42; P < 0.001) between SI and body weight. Mean Sg also changed significantly over time; 1.5-, 2.0-, and 1.7-fold increases (compared with

### Table 1

<table>
<thead>
<tr>
<th>Week of treatment</th>
<th>Pretreatment (n = 17)</th>
<th>16 (n = 18)</th>
<th>32 (n = 18)</th>
<th>48 (n = 18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline glucose (mg/dL)</td>
<td>74.3 ± 5.9</td>
<td>72.0 ± 3.3</td>
<td>72.8 ± 6.1</td>
<td>74.3 ± 8.9</td>
<td>0.379</td>
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<tr>
<td>Baseline insulin (mU/L)</td>
<td>9.3 ± 0.6</td>
<td>6.0 ± 3.0*</td>
<td>7.4 ± 3.1</td>
<td>7.6 ± 4.7</td>
<td>0.046</td>
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<tr>
<td>SI (x 10⁻⁴ [L/min•μU/mg])</td>
<td>1.8 ± 1.0</td>
<td>3.3 ± 1.4*</td>
<td>4.4 ± 2.0*</td>
<td>3.5 ± 0.9* &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Sg (x 10⁻¹ [%/min])</td>
<td>1.5 ± 0.6</td>
<td>2.2 ± 0.7*</td>
<td>3.0 ± 1.1*</td>
<td>2.5 ± 1.1* &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>AIRg (mU/L•min)</td>
<td>365 ± 220</td>
<td>276 ± 109*</td>
<td>257 ± 107*</td>
<td>319 ± 147 0.005</td>
<td></td>
</tr>
<tr>
<td>DI (x 10⁻¹)</td>
<td>5.0 ± 4.1</td>
<td>9.2 ± 4.8*</td>
<td>10.1 ± 4.1*</td>
<td>11.2 ± 6.0* 0.001</td>
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</tr>
</tbody>
</table>

*Within a row, mean value differs significantly from pretreatment mean value as determined via ANOVA.

### Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Week of treatment</th>
<th>Pretreatment</th>
<th>16</th>
<th>32</th>
<th>48</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose concentration</td>
<td>6 (1–9)</td>
<td>4 (0–8)</td>
<td>4 (1–7)</td>
<td>4 (1–9)</td>
<td>5 (0–9)</td>
<td></td>
</tr>
<tr>
<td>Serum insulin concentration</td>
<td>26 (7–57)</td>
<td>26 (4–62)</td>
<td>29 (18–69)</td>
<td>34 (5–29)</td>
<td>29 (4–89)</td>
<td></td>
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<tr>
<td>Sg</td>
<td>27 (2–64)*</td>
<td>20 (6–36)</td>
<td>33 (10–67)</td>
<td>12 (9–42)</td>
<td>26 (2–67)</td>
<td></td>
</tr>
<tr>
<td>AIRg</td>
<td>33 (28–41)*</td>
<td>27 (8–38)</td>
<td>17 (8–29)</td>
<td>17 (2–8)</td>
<td>23 (2–41)</td>
<td></td>
</tr>
</tbody>
</table>

*Coefficients of variation calculated for each horse at each testing point; reflects variability between measurements obtained daily on 3 consecutive days.

See Table 1 for remainder of key.
the pretreatment mean value) were detected at weeks 16, 32, and 48, respectively (Table 1). However, Sg was not correlated with body weight ($r = 0.06; P = 0.611$). Mean AIRg significantly ($P < 0.001$) decreased over time. Means differed significantly from the pretreatment mean value at 16 and 32 weeks, and mean AIRg was 24%, 30%, and 13% lower than the pretreatment mean value at 16, 32, and 48 weeks, respectively. A positive correlation ($r = 0.69; P < 0.001$) between AIRg and body weight was detected, and AIRg was negatively correlated with SI ($r = -0.31; P = 0.008$). Disposition index significantly increased during L-T$_4$ treatment ($P < 0.001$).

Overall mean CV values were 5% and 29% for pre-test plasma glucose and serum insulin concentrations, respectively, and were 33%, 26%, and 23% for SI, Sg, and AIRg, respectively (Table 2). Mean CV values for each testing week represented the repeatability of the FSIGT test procedures performed on 3 consecutive days and assessment of each set of data via minimal model analysis.

**Discussion**

In horses that were known to be euthyroid on the basis of serum thyroid hormone concentrations andthyrotropin-releasing hormone response test results, L-T$_4$ administration was associated with significant weight loss and alterations in glucose dynamics. During treatment, both SI and Sg significantly increased, whereas AIRg significantly decreased.

A dosage of 48 mg of L-T$_4$/d was selected for use in the present study after reviewing results of our previous investigation and considering the dosages recommended by equine practitioners. A fixed amount of 48 mg of L-T$_4$ provided in 4 teaspoons of powder was chosen to reflect common dosing practices and simplify procedures for long-term administration of the drug. However, variability in body weight among the study horses and within each horse over the 48-week study period resulted in administration of L-T$_4$ at a range of dosages from 87 to 121 µg/kg. Administration of more consistent dosages may have reduced variability in responses among individual study horses.

Results of a previous investigation by our group indicated that L-T$_4$ induces weight loss in horses, but to our knowledge, the present study is the first to examine the effects of this drug during a 48-week period. Among the 6 horses, maximum mean weight loss (10%) relative to the pretreatment mean value was detected at 16 weeks, and 5% weight loss was still evident at the completion of the study. It is assumed that L-T$_4$ induces weight loss by increasing basal metabolic rate and reducing body fat stores. Body fat mass decreased by approximately 50% in rats treated with T$_4$, and this finding was accompanied by increases in feed intake, caloric expenditure, and oxygen consumption. Weight loss and hyperphagia were also detected in rats treated with 375 µg of L-T$_4$/kg, and treatment decreased epididymal fat pad weight and adipocyte size.

Changes in body weight may have been confounded by differences in feed intake and environmental conditions during the study. Feed intake was not controlled and could not be measured because horses were turned out on pasture each day and given access to hay in round bales. Variation in the amount or composition of the hay provided or grass consumed on pasture may have also influenced the horses’ weight. Unfortunately, the feeds provided in the present study were not analyzed; thus, effects of diet on body weight and glucose dynamics in the study horses cannot be assessed. It should also be recognized that the treatment period began in November; therefore, measurements obtained at 16 weeks could reflect colder ambient temperatures and differences in the quality and quantity of grass consumed. These factors may also explain the relative increase in body weight after this time point. Horses may have gained weight as the pasture grass improved in quality and abundance during the spring and summer months. Inclusion of a control group would have helped to address these questions, but control horses were not included because of the length of the study and financial constraints.

Plasma glucose concentrations evaluated before FSIGT tests were not affected by treatment in our study, but serum insulin concentrations significantly decreased over time. Lower baseline serum insulin concentrations were also detected in dogs when 100 µg of L-T$_4$/kg was administered via SC injection every day for 10 days. However, the same dogs had higher baseline blood glucose concentrations and blunted insulin responses to glucose infusions. It was concluded that pancreatic insulin secretion decreases but that SI remains unchanged when L-T$_4$ is administered to dogs. In rats, the responses to L-T$_4$ have varied according to the dosage administered and duration of treatment. No differences in baseline plasma glucose and serum insulin concentrations were detected when rats were treated with 375 µg of L-T$_4$/kg/d for 10 days, but glucose concentrations decreased and insulin concentrations increased in rats treated with the same dosage for 30 days. In contrast, both plasma glucose and serum insulin concentrations increased significantly from pretreatment values when L-T$_4$ was administered daily to rats at a dosage of 500 µg/kg for 7 days. Results of those studies illustrate that responses to L-T$_4$ vary considerably among animal species and depend on the specific treatment selected.

In the present study, L-T$_4$ administration was associated with a > 2-fold increase in SI in euthyroid horses. In another study, we detected a 2.5-fold increase in SI after L-T$_4$ was administered to horses at dosages ranging from 24 to 96 mg of L-T$_4$/d for 8 weeks, but it was not clear whether SI would remain increased if treatment were extended. Results of the present study have indicated that effects of L-T$_4$ on SI have a duration > 8 weeks; however, it still cannot be determined whether this change should be attributed to weight loss or to the treatment itself.

Insulin sensitivity improves with weight loss in overweight and obese humans, but lean individuals have not been examined to our knowledge. Obese humans without diabetes mellitus lost 8.3 ± 4.2 kg (8% of initial body weight) and mean SI increased by 63% when they received a weight-loss diet for 60 days. A 57% improvement in SI was also detected in overweight or obese older men who lost 10% of body weight over a 3-month period. There is also evidence that thyroid
hormones exert direct effects on glucose uptake and SI. A 3.6-fold increase in glucose uptake was detected when cultured 3T3-L1 adipocytes were exposed to T3 for 3 days, and a 44% increase in insulin-stimulated glucose uptake was detected in adipocytes harvested from rats treated with L-T4 for 7 days. Basal glucose uptake was also approximately 2-fold higher in skeletal muscle collected from rats that were treated with 375 µg of L-T3/kg/d for 10 to 30 days, compared with samples from untreated control rats.

We have previously reported that during L-T4 treatment, there was an increase in the abundance of GLUT4 mRNA within adipose tissues from 4 of the 6 horses included in the present study. Detection of increased GLUT4 gene expression in adipose tissue collected from horses after long-term L-T4 treatment suggests that the abundance of GLUT4 protein also increases, which might increase SI. This theory is supported by results of other studies. Matthaï et al. determined that insulin-stimulated glucose uptake was 44% higher in adipocytes harvested from rats treated with L-T4 for 7 days, compared with adipocytes from untreated rats, and that this was accompanied by an increase in GLUT4 protein abundance. In a study by Romero et al., quantities of GLUT1 and GLUT4 proteins increased by 87% and 90%, respectively, in cultured 3T3-L1 adipocytes that were exposed to T3 for 3 days. However, it has also been reported that L-T4 lowers the density of insulin receptors within adipocyte plasma membranes in rats, indicating that higher GLUT4 gene expression may reflect a compensatory response.

Glucose effectiveness represents uptake of glucose into cells that is mediated by glucose itself. Pretreatment mean Sg values in the horses of the present study compared favorably with reported mean values of 1.43 X 10⁻⁴/min and 1.59 X 10⁻³/min for nonobese or moderately obese Thoroughbred geldings, respectively. In the horses of our study, mean Sg increased over time and was accompanied by higher SI, which suggests that glucose uptake into tissues is enhanced via both insulin-dependent and insulin-independent pathways in horses treated with L-T4.

In the present study, mean AIRg was 13% to 30% lower during treatment, which compared favorably with the 19% decrease in this variable detected in overweight or obese older men that lost a mean value of 10% of body weight over 3 months. Insulin sensitivity improved by 57% and DI increased by 33% in those same men. The increased DI value indicated overall improved beta-cell function following weight loss, but the mechanism responsible for this effect remains unknown. This is the opposite response to the increase in AIRg associated with compensated IR in ponies. Insulin-resistant ponies develop hyperinsulinemia as SI decreases and AIRg increases to maintain glucose delivery to tissues. In obese humans without diabetes mellitus that have IR, high AIRg values are also detected and AIRg decreases as SI increases with weight loss.

Disposition index increased throughout the study period even as AIRg decreased. The DI reflects pancreatic responsiveness after accounting for SI and is calculated by multiplying AIRg by SI. In insulin-resistant humans, DI is examined to assess the ability of pancreatic beta cells to respond to decreased SI by increasing endogenous insulin secretion and low DI values predict type 2 diabetes mellitus. In the horses of the study reported here, higher DI values reflected an increase in SI that was of greater magnitude than the reduction in AIRg, which is consistent with the response detected in humans following weight loss.

In our study, baseline plasma glucose concentrations assessed before the FSIGT tests were repeatable; mean within-horse CV values were approximately 5%. In contrast, within-horse variability was much higher for baseline serum insulin concentrations and the CV value was 29% for the entire study. This finding may be attributed to variable feed intake because the horses were fed grass hay ad libitum before and during FSIGT test procedures. Horses were permitted to eat hay as a means of reducing stress and to facilitate collection of samples. It is known that IR is induced when healthy horses undergo a stressful event prior to glucose tolerance testing. It has also been shown that the stress associated with transport increases blood glucose and insulin concentrations in donkeys by 130% and 275%, respectively. The decision to feed hay was also based on the assumption that the particular feed used elicits a low glycemic response.

Higher within-horse variability in serum insulin concentrations may be the result of pulsatile secretion of insulin from the pancreas. In another study, mean serum insulin concentrations fluctuated throughout the day in 4 geldings from which samples were collected every 15 to 60 minutes during 24 hours of feed deprivation. In donkeys that were deprived of feed for 72 hours, hourly fluctuations in serum insulin concentrations were detected. These results suggest that insulin secretion from the pancreas is pulsatile in horses, as it is in humans and mice. Measurement of serum insulin concentrations every 30 seconds during a 50-minute period in humans revealed successive peak and trough concentrations, and secretory pulses occurred at intervals of approximately 5 minutes. It is suggested that multiple baseline samples should be collected to assess resting insulin concentrations in horses in future studies.

In the present study, assessment of the repeatability of the FSIGT test and minimal model analysis procedures revealed that the highest mean CV (33%) was associated with SI, compared with 26% and 23% for Sg and AIRg, respectively. These differences may be a result of greater variability in serum insulin concentrations or reflect differences in model fitting. The repeatability of these procedures has been evaluated by testing 6 horses on 2 occasions over a 2-week period; mean interday CVs of 24% (range, 9% to 35%), 26% (range, 13% to 40%), and 12% (range, 7% to 21%) were reported for SI, Sg, and AIRg, respectively. Results of the previous study and that of this report should be considered when designing future experiments that employ similar methods; within-horse variability of approximately 30% must be accounted for.

Results of the present study have indicated that L-T4 administration significantly lowered body weight...
and raised SI in euthyroid horses, but it is possible that alterations in diet or season may have confounded results. Nevertheless, L-T₄ may be useful for the treatment of obesity and IR in horses, but further studies are required.

c. Dextrose 50% injection, Abbott Laboratories, North Chicago, Ill.
d. Humulin R, Eli Lilly and Co, Indianapolis, Ind.
e. Glucose, Roche Diagnostic Systems Inc, Somerville, NJ.
f. Cobas Mira, Roche Diagnostic Systems Inc, Somerville, NJ.
g. Coat-A-Count insulin, Diagnostic Products Corp, Los Angeles, Calif.
h. MinMod Millenium, version 6.10, Raymond Boston, University of Pennsylvania, Kennet Square, Pa.
i. Stata 9.2, Stata Corp, College Station, Tex.
j. PROC MIXED, SAS, version 9.1, SAS Institute Inc, Cary, NC.

References