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Original scientific article

Insulin dosing affects plasma levels of biochemical parameters in a time-dependent manner in Sprague-Dawley rats

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Summary

Changes in levels of various biochemical blood parameters are used as indicators of metabolic effects or potential toxicity when performing non-clinical safety studies of new drug candidates in rats. Additionally, since biochemical blood parameters are often affected during safety testing of new insulin analogues the effect of insulin dosing on these parameters was investigated. Non-diabetic rats were dosed with either vehicle or insulin once daily for 28 days. On Day 28, biochemical blood parameters as well as insulin exposure were measured, at two hour intervals during a 24 h period, to investigate time-dependent as well as time-independent changes. Insulin dosing lowered plasma glucose level for 4 h, corresponding to the peak plasma insulin level. Chronic insulin dosing increased food consumption and bodyweights. Additionally, plasma urea as well as CK and LDH levels increased. Hyperphagia was most likely driven by hypoglycaemia thereby also increasing body weight through insulin-stimulated fatty acid uptake into adipose tissue. Increased urea, CK and LDH levels, suggests that the return to normoglycaemia was driven mainly by increased hepatic gluconeogenesis, as reflected by increased ureagenesis and skeletal muscle proteolysis (increased CK and LDH). A better understanding of insulin-induced changes to biochemical blood parameters may aid the interpretation of changes in these parameters in non-clinical safety studies with new drugs

Introduction

Changes in levels of biochemical blood parameters are used to evaluate the metabolic consequences or potential toxicity of new drug candidates when performing repeat-dose toxicity studies as part of the safety assessment (*European Medicines Agency, 2010*). According to regulatory guidelines, toxicological evaluation of new antidiabetic drug candidates are conducted using non-diabetic animals (*US Food and Drug Administration, 2008; European Medicines Agency, 2001*). The rat is often the rodent species of choice based on its pharmacological responsiveness and availability of extensive historical control data.

When dosing non-diabetic rats with insulin, the resulting hypoglycaemia activates counter-regulatory responses, such as increased food consumption and bodyweight (*May & Beaton, 1968*), increased release of certain hormones (e.g. glucagon) (*Cryer, 1993*) and increased hepatic glucose production (*Gazola et al., 2007*), which are typically reflected in changes of biochemical blood parameters. All of these changes

challenge differentiation between effects caused by the hypoglycaemia and potential off-target effects. Differentiation is typically aided by including a comparator group dosed with human insulin, serving as a reference (*European Medicines Agency, 2001*). Therefore, we have chosen human insulin for the present study. The metabolic effects of insulin dosing and the counter-regulatory response may potentially be influenced by the nocturnal feeding pattern of rats, and both may affect several parameters involved in glucose and fat metabolism such as liver glycogen stores (*Marrino et al., 1987*). It is important to have detailed knowledge of the normal metabolic counter-regulatory changes to hypoglycaemia in non-diabetic animals when interpreting results from non-clinical safety studies. Thus, an understanding of time-dependent changes in metabolism following insulin dosing is pivotal and would allow for improved study design and timing of blood sampling, as well as aiding interpretation of any biochemical changes, in non-clinical safety studies in rats.

The aim of the present study was to investigate time-dependent effects of repeated insulin dosing, during the early light period, in non-diabetic rats on levels of biochemical blood parameters in relation to exposure and blood glucose level.

This was done using insulin dosing for 28 days, causing recurrent daily hypoglycaemia, and evaluating the effect on biochemical parameters in blood taken every two hours following the end of dosing.

Materials & Methods

Animals

Male and female Sprague-Dawley (Crl:CD (SD), Charles River Deutschland, Germany) rats approximately 7 weeks old were used (n=60/sex, allowing for n=5/sampling time-point/sex, see below). Rats were randomly allocated to groups (2 groups/sex, n=30/group/sex) stratified for sex and body weight so that body weight was similar between groups (mean±SD: control males: 240.7±8.8g, dosed males: 240.4±7.3g, control females: 166.8±6.8g, dosed females: 166.8±8.6g). They were housed in transparent Macrolone type IV cages (floor area 1800 cm^2, height 31 cm, 2-3 animals/cage) with Aspen wood shavings, wooden blocks, paper strand material and plastic shelters. Each sex and group was housed separately, with free access to water and a standard complete pelleted rodent diet. Twice weekly the animals were offered a small amount of cereal grain and maize. Animals were acclimatized for approximately 7 days before the start of dosing on Day 1. The facility was illuminated with a twelve-hour light/dark cycle (lights on 6:00 AM) with controlled temperature, humidity and air change (18-24˚C, relative humidity 30-70%, air change 8-15 times/hour). All procedures involving live animals were performed under the Animal Licence authorized by the Danish Animal Experimentation Inspectorate and according to local standard operating procedures, Good Documentation Practice and OECD guidance on Humane Endpoints for Experimental Animals in Safety Studies.

Study design

Animals were dosed subcutaneously once daily (at 8:00-10:00 AM) for 28 days with either Neutral Protamine Hagedorn (NPH) recombinant human insulin (65 nmol/kg/day, NPH-Group) (Insulatard®, Novo Nordisk A/S, Denmark) or vehicle (CTRL-Group) based on the body weight of each animal. Animals were dosed group-wise, with the same order at each dosing so as to allow for equal time between dosing. The vehicle was an isotonic solution of glycerol 16.0 mg/ml, phenol 0.65 mg/ml, m-cresol 1.5 mg/ml, disodium phosphate dihydrate 2.40 mg/ml and had a pH of approximately 7.3. Bodyweight and food consumption were measured twice weekly. On the last day of dosing (Day 28) blood samples (1.2 ml, sublingual vein) were obtained every two hours for a period of 24 hours, with first samples taken pre-dosing. Animals were sampled in the same sequence as for dosing. Each animal was sampled on two occasions 12 h apart (5 animals per sex/time-point) and terminated after the last sample. Each sampling took <30 seconds and blood was sampled into microtubes containing lithium heparin. The tubes were gently inverted at least 10 times, centrifuged (2000 g, 10 minutes, 4°C) and the plasma separated and frozen (-20°C) in plastic tubes within 30 min after centrifugation.

Levels of the following biochemical parameters were quantified in plasma using a Pentra C400 Clinical Chemistry benchtop analyser (HORIBA, Ltd., Japan): glucose, triglyceride, cholesterol, urea, creatinine, total protein, albumin, globulin, albumin/globulin (A/G) ratio, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine kinase (CK), sodium, potassium, calcium, inorganic phosphate and chloride.

Levels of NPH insulin in plasma samples were quantified using a commercially available ELISA kit (K6219, DAKO, Denmark) according to the manufacturer's instructions. The lower limit of quantifica-

tion (LLOQ) was 10 pmol/L. Samples were analysed in duplicate and mean concentrations were reported.

Statistics

Each sex was analysed separately. Bodyweight and food consumption data were analysed using a two-way repeated measures analysis of variance (ANOVA) (effect of insulin dosing and time), followed by a *post hoc* Sidak's multiple comparisons in case of an overall significant effect of dosing. All plasma measurement data from both groups (CTRL and NPH) were analysed by a two-way ANOVA to test for overall effect of insulin dosing, and to determine if it was dependent on time after dosing (interaction). In case of an overall significant effect of dosing, data were further analysed with *post hoc* Sidak's multiple comparisons. Additionally, bodyweight and food consumption data were analysed using repeated measures ANOVA with *post hoc* Sidak's multiple comparisons. Unless otherwise stated, results are given as mean±SD. A p-value ≤0.05 was considered statistically significant.

Results

Animals

No clinical signs related to dosing of insulin (NPH-Group) or vehicle (CTRL-Group) were observed. Bodyweights were generally significantly increased from Day 14 in the NPH-Group compared to CTRL-Group in both males and females (Fig. 1). On Day 28, bodyweights were increased by 4% and 3% in NPH- versus CTRL-Group for males and females, respectively. Food consumption (data not shown) in females was significantly increased in NPH- versus CTRL-Group (p=0.0318) generally by 6-12% throughout the study. Food consumption in males was generally, but not significantly, 7-10% higher in NPH- versus CTRL-Group (p=0.1072), with an indication of a possible time-dependent effect of insulin dosing (p=0.0661).

Plasma NPH insulin levels: The NPH insulin plasma concentration was <LLOQ in all CTRL samples (data not shown). Plasma NPH insulin levels in the NPH-Group are shown in Fig. 2A+B. Maximum exposure level was achieved 2 h after dosing, followed by a gradual decline with levels approaching LLOQ 10-12 h after dosing.

Effect of insulin dosing on levels of biochemical blood parameters

Overall changes of insulin dosing on biochemical parameters are summarized in Table 1.

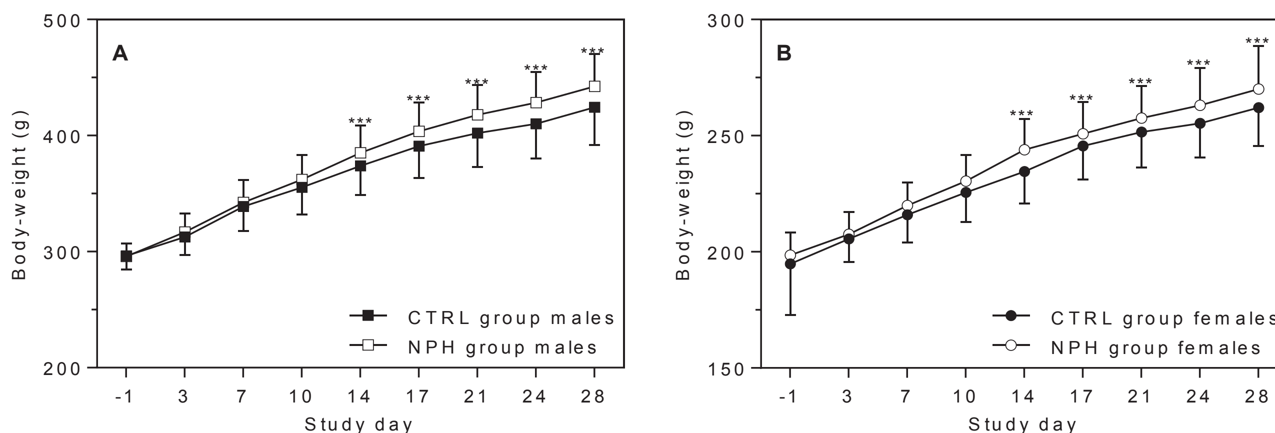

Figure 1 Body-weights

Mean±SD. For each sex: n=30/time-point per group. A) males, B) females. FBodyweight was measured twice weekly. Levels were increased from day 14 onwards in both males and females in NPH groups versus CTRL groups. Effect of insulin dosing and time-point on body weight was evaluated using a 2-way ANOVA for each sex separately. As there was a time-dependent effect of insulin dosing, a *post hoc* Sidak's multiple comparisons test was performed for each time-point separately, where ***p<0.001 versus CTRL group.

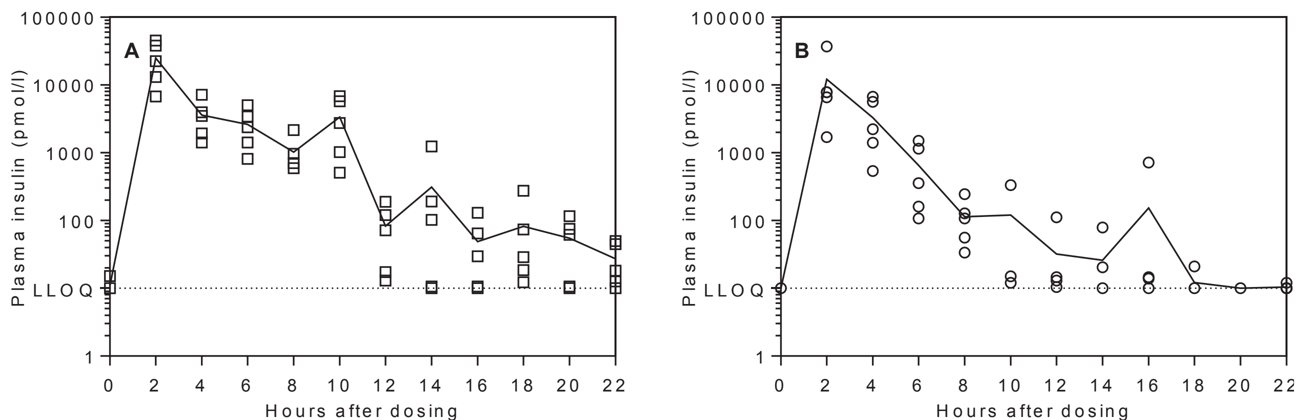

Figure 2 Plasma NPH insulin levels
Mean (line) and individual (symbols) values. n=5/time-point for each sex. Plasma NPH insulin levels in: A) NPH-Group males and B) NPH-Group females. Time-point zero represents a sample taken prior to dosing with insulin. Levels measured as <LLOQ are depicted as the nominal value 10 pmol/l (LLOQ).

Table 1. Summary of effect of insulin-dosing on plasma parameter levels
A) males, B) females. For each sex: n=4-5/time-point per group. Statistical values reported are from a 2-way ANOVA (factors: group and time-point). P-values refer to the effect of insulin-dosing (group) unless there was a time-dependent effect of insulin dosing, in which case this p-value is reported. DF, degrees of freedom. NC, no change. +, increased. ÷, decreased. A/G, albumin/globulin. ALT, alanine aminotransferase. AST, aspartate aminotransferase. ALP, alkaline phosphatase. CK, creatine kinase. LDH, lactate dehydrogenase.

A. Males

Parameter	Change	P-value	F-value	DF
Glucose	÷	<0.0001[a]	12.81	11
Cholesterol	NC	<0.0001[a]	4.263	11
Triglyceride	÷	0.0005	13.02	1
Urea	+	0.0231[a]	2.158	11
Creatinine	+	0.0454[a]	1.924	11
Total protein	÷	0.0200[a]	2.208	11
Albumin	÷	0.0069[a]	2.56	11
Globulin	÷	<0.0001	23.05	1
A/G ratio	÷	0.0254	5.154	1
ALT	NC	0.3454	0.899	1
AST	NC	0.1014	2.736	1
ALP	÷	0.0063	7.797	1
CK	+	0.0299	4.861	1
LDH	NC	0.9563	0.003012	1
Calcium	÷	0.0018	10.32	1
Chloride	+	0.0047[a]	2.69	11
Sodium	÷	<0.0001[a]	5.012	11
Potassium	+	0.0106[a]	2.42	11
Inorganic phosphate	NC	0.3208	0.9960	1

[a]Interaction between effect of dosing and time-point.

B. Females

Parameter	Change	P-value	F-value	DF
Glucose	÷	<0.0001[a]	10.17	11
Cholesterol	÷	0.00243	0.9367	1
Triglyceride	NC	0.1971	1.687	1
Urea	+	0.0080[a]	2.511	11
Creatinine	NC	0.8915	0.0187	1
Total protein	÷	0.0035	8.946	1
Albumin	÷	0.0004	13.48	1
Globulin	NC	0.1599	2.006	1
A/G ratio	÷	0.0096[a]	3.822	11
ALT	NC	0.9406	0.005604	1
AST	NC	0.6617	0.1927	1
ALP	NC	0.8415	0.04023	1
CK	+	0.0134[a]	2.342	11
LDH	+	0.0363[a]	2.002	11
Calcium	NC	0.2606	1.280	1
Chloride	NC	0.5360	0.3858	1
Sodium	÷	0.0114	6.654	1
Potassium	+	0.0059[a]	2.612	11
Inorganic phosphate	+	0.0061[a]	2.600	11

[a]Interaction between effect of dosing and time-point.

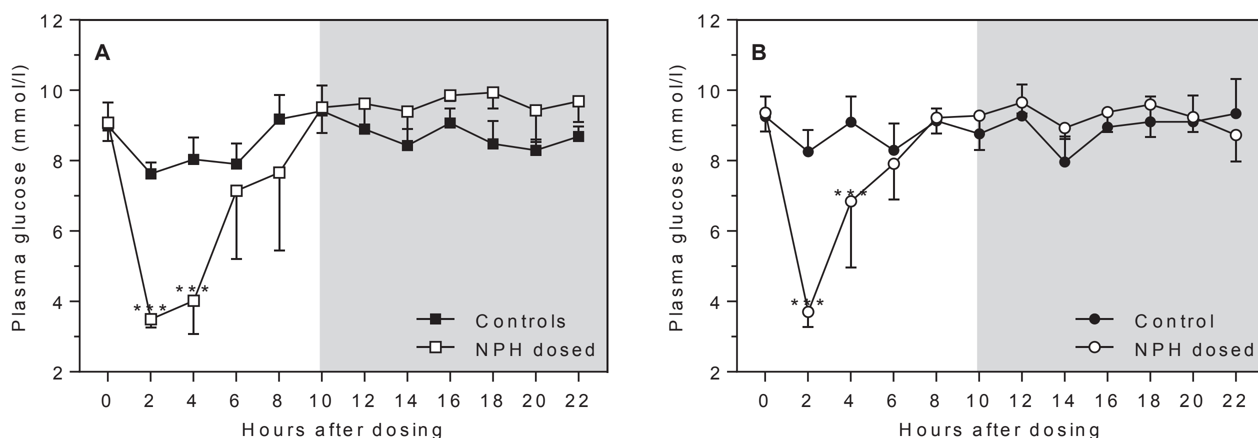

Figure 3 Effect of insulin dosing on plasma glucose levels
Means±SD. For each sex: n=5/time-point per group. Grey box indicates dark period. Plasma glucose levels in: A) males, and B) females. Time-point zero represents a sample taken prior to s.c. dosing with NPH insulin. 2-way ANOVA showed a time-dependent effect of NPH dosing, consequently a *post hoc* Sidak's multiple comparisons was performed for each time-point separately: ***p<0.001 versus the CTRL group.

Glucose: Insulin dosing affected plasma glucose levels significantly in a time-dependent manner i.e. interaction between effect of group and time after dosing (p<0.0001 for either sex). After insulin dosing at time-point zero a significant plasma glucose lowering effect was seen for 4 h after dosing in males and females (Fig. 3A+B), with maximal effect on blood glucose levels 2 h after dosing, after which the effect tapered off with levels similar to controls approximately 6 h after dosing.

Cholesterol: There was no overall effect of insulin dosing or time-point on plasma cholesterol levels in males, whereas dosing caused an overall significant increase in cholesterol in females (p=0.0243) (Fig. 4A+B). There was no significant difference from controls at any of the individual time-points.

Triglyceride: In males, plasma levels were significantly decreased by insulin dosing (p=0.0005), as well as significantly affected by time-point (p=0.0349). *Post hoc* analysis revealed significantly lower levels 2 h and 20 h after dosing (p=0.0278 and p=0.0332) (Fig. 4C). In females, plasma triglyceride levels were only significantly affected by time-point (p=0.0001), and seemed to be lower during the early light period independent of dosing (Fig. 4D).

Urea: Plasma levels were significantly affected by insulin dosing in a time-dependent manner in both males (interaction, p=0.0231) and females (interaction: p=0.0080). Levels seemed to be increased for up to 10 h after insulin dosing in males and females (Fig. 4E+F). However, *post hoc* analysis did not reveal any significant differences between groups at individual time-points except for 6 h after dosing in females.

Creatinine: Plasma levels were not affected by insulin dosing in females; however, in males they were significantly affected in a time-dependent manner (p=0.0454), where levels generally seemed to be increased from 10 h after dosing (Fig. 4G+H).

Total protein, albumin, and globulin: Insulin dosing significantly affected total protein levels in males in a time-dependent manner (p=0.0200), generally with lowered total protein levels from 12 h after dosing, although only significantly from the controls at 14 h (p=0.0007) as well as 2 h (p=0.0296) after dosing (Fig. 5A). In females, total protein levels were significantly affected by both insulin dosing (p=0.0035) and time-point (p=0.0184), generally with lowered levels but at the individual time-points this difference was only significant 16 h after dosing (p=0.0205) (Fig. 4B). In males, albumin levels were significantly affected in a time-dependent manner (p=0.0069), with significantly decreased levels immediately prior to dosing (p=0.0262) and at 2 h (p=0.0090) and 14 h (p=0.0056) after dosing (Fig 5C). In females, an overall decrease in albumin levels was seen following insulin dosing (p=0.0004) (Fig. 5D). Additionally, levels were significantly affected by time-point (p=0.0415), although only significantly lower than controls at 4 h after dosing (p=0.0342). Globulin levels were significantly affected by both insulin dosing in males (p<0.0001), with decreased levels in the NPH group, and time-point (p=0.0383), whereas levels in females were only significantly affected by time-point (p=0.0073) (Fig. 5E+F). *Post hoc* analysis revealed significantly decreased levels 14 h and 16 h after dosing in males

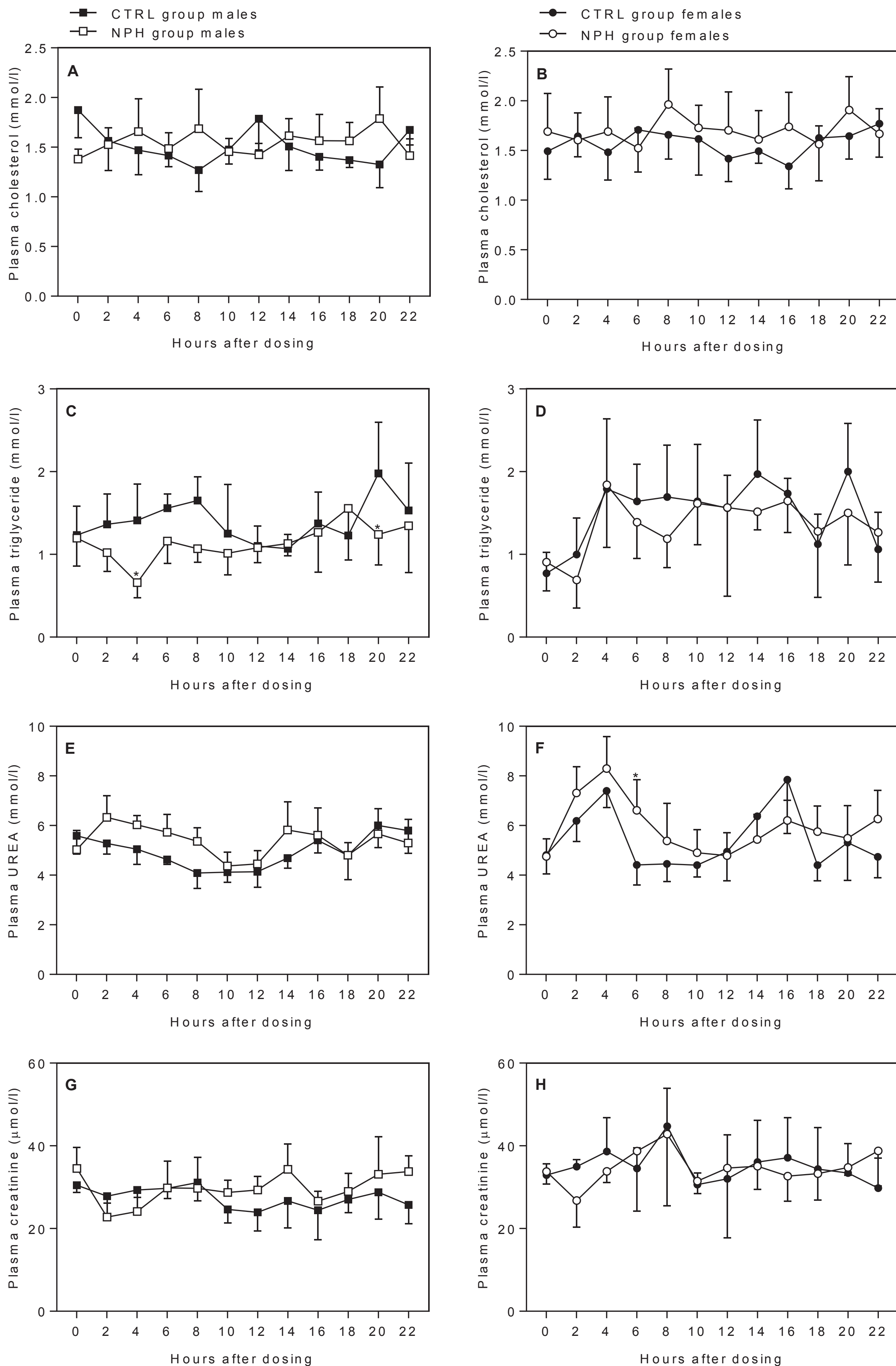

Figure 4 Effect of insulin dosing on plasma levels of cholesterol, triglyceride, urea and creatinine
Means±SD. For each sex: n=4-5/time-point per group. Left column: males, right column: females. Time-point zero represents a sample taken prior to s.c. dosing with NPH insulin. In case of an effect of NPH dosing, a *post hoc* Sidak's multiple comparisons test was performed for each time-point separately: *p<0.05 versus the CTRL group.

and females, respectively. The A/G (albumin/globulin) ratio was significantly affected by insulin dosing in males (p=0.0254), with an increased ratio, whereas in females it was significantly affected in a time-dependent manner (p=0.0096), with decreased levels up to 8 h after dosing (Fig. 5G+H), although with no significant difference from controls at the individual time-points.

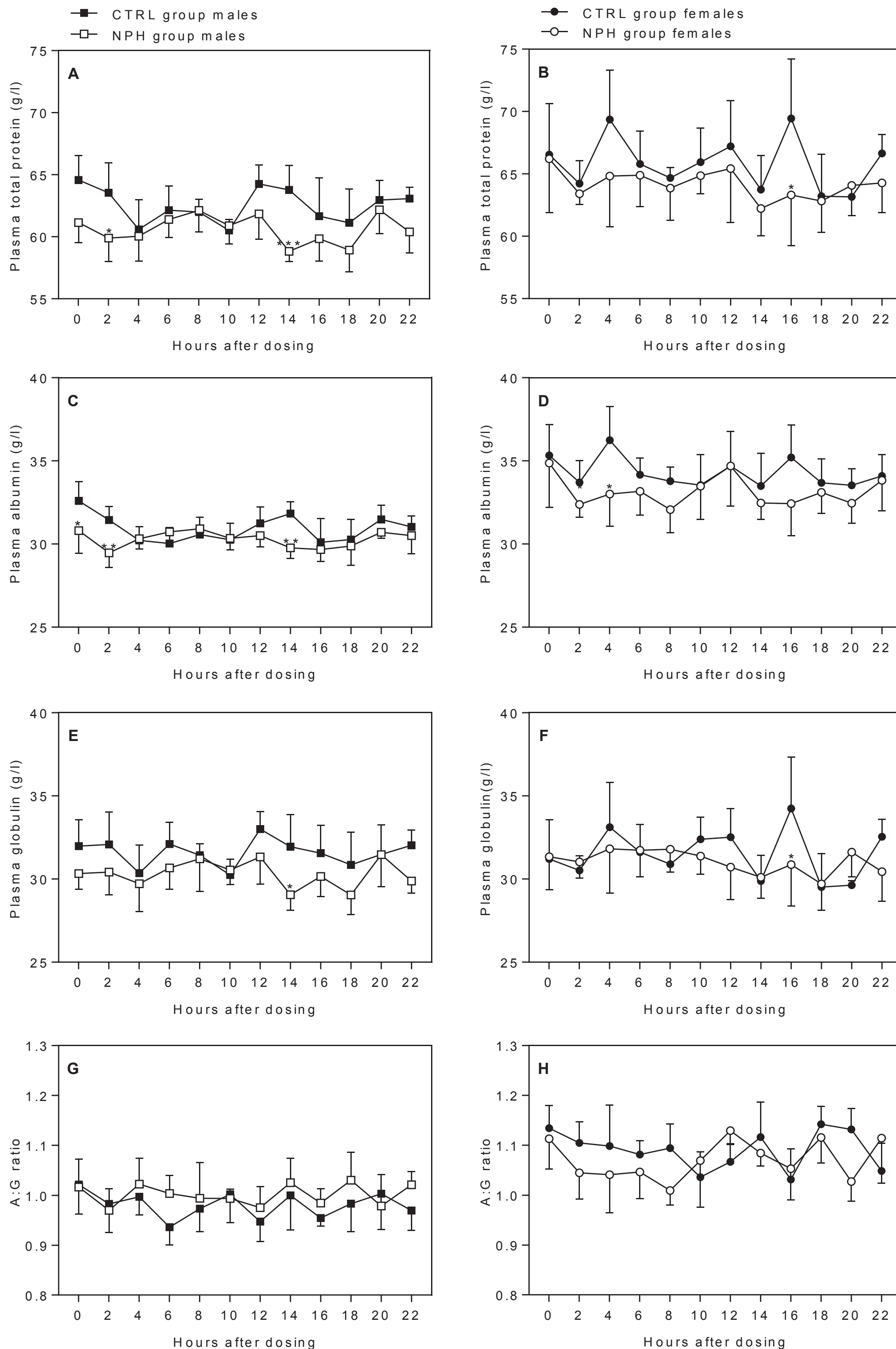

Figure 5 Effect of insulin dosing on plasma levels of proteins
Means±SD. For each sex: n=4-5/time-point per group. Left column: males, right column: females. Time-point zero represents a sample taken prior to s.c. dosing with NPH insulin. In case of an effect of NPH dosing a *post hoc* Sidak's multiple comparisons test was performed for each time-point separately: *p<0.05 versus the CTRL group.

ALT, AST and ALP: Insulin dosing did not affect ALT (alanine aminotransferase) or AST (aspartate aminotransferase) levels significantly in males and females, whereas AST levels were significantly affected by time-point in females (p=0.0029) (Fig. 6A-D). ALP (alkaline phosphatase) levels in males were significantly decreased by insulin dosing (p=0.0063) (Fig. 6E+F), but with no significant differences at the individual time-points; no effects were seen in females.

CK: Plasma levels of CK (creatine kinase) were significantly affected by insulin dosing (p=0.0299) and time-point (<0.0001) in males, with increased levels in the NPH-dosed group primarily at 8 h, 14 h and 22 h (Fig. 6G), although not statistically significantly different from controls at the individual time-points. In insulin-dosed females levels were significantly increased in a time-dependent manner (p=0.0134), with a pronounced significant increase at 6 h (p=0.0001) (Fig. 6H).

LDH: Plasma levels of LDH (lactate dehydrogenase) were only significantly affected by time-point in males (p<0.0001) (Fig. 5I), whereas levels were significantly affected by insulin dosing in a time-dependent manner in females (p=0.0363), which generally were decreased, but with no significant changes at any of the individual time-points (Fig. 6J).

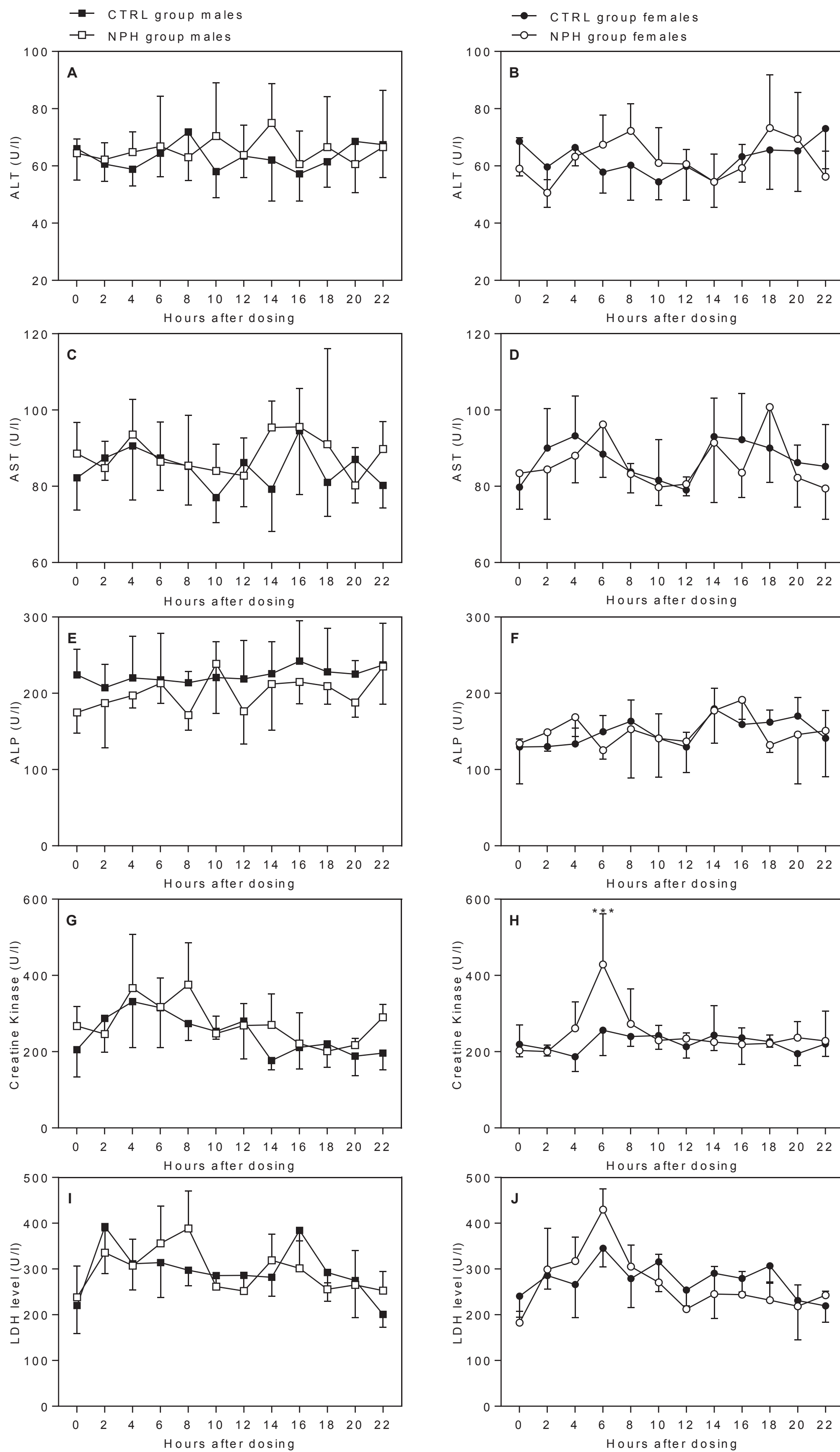

Figure 6 Effect of insulin dosing on plasma levels of enzymes
Means±SD. For each sex: n=4-5/time-point per group. Left column: males, right column: females. Time-point zero represents a sample taken prior to s.c. dosing with NPH insulin. In case of an effect of NPH dosing a *post hoc* Sidak's multiple comparisons test was performed for each time-point separately: there was no difference at individual time-points versus the CTRL group for any of the enzymes.

Calcium: In males, plasma levels of calcium were significantly affected by time-point ($p=0.0010$) and insulin dosing ($p=0.0018$) approaching a time-dependent effect ($p=0.0548$) (Fig. 7A); levels were significantly lower 2 h ($p=0.0107$) and 22 h ($p=0.0376$) after dosing. In females, calcium levels were only significantly affected by time-point ($p<0.0001$), with maximum values at 6 h and minimum values at 16 h (Fig. 7B).

Chloride: In males, plasma levels of chloride were significantly affected by insulin dosing in a time-dependent manner (0.0047), with significantly increased levels 2 h after dosing ($p=0.0023$) (Fig. 7C). Chloride levels were not affected by insulin dosing in females, whereas there was a significant effect of time-point ($p<0.0001$) (Fig. 7D), with maximum values at 2 h and minimum values at 16 h.

Sodium: In males, insulin dosing significantly affected sodium levels in a time-dependent manner ($p<0.0001$), with a significant decrease in levels 14 h after dosing ($p<0.0001$) and 24 h after dosing just prior to the next dose ($p=0.0014$) (Fig. 7E). Insulin dosing also significantly affected plasma sodium levels in females ($p=0.0114$), with a significant decrease at 22 h ($p=0.0166$) (Fig. 7F).

Potassium: Insulin dosing significantly affected plasma levels of potassium in a time-dependent manner in both males ($p=0.0106$) and females ($p=0.0059$), with a significant increase 14 h and 6 h after dosing in males ($p=0.0013$) and females ($p=0.0002$), respectively (Fig. 7G+H). The pattern of changes was approximately opposite to that observed for sodium.

Inorganic phosphate: Plasma levels were only significantly affected by time-point ($p<0.0001$) and not by insulin dosing in males, whereas in females levels were significantly affected in a time-dependent manner ($p=0.0061$) with significantly increased levels 2 h after dosing ($p=0.0309$) (Fig. 7I+J).

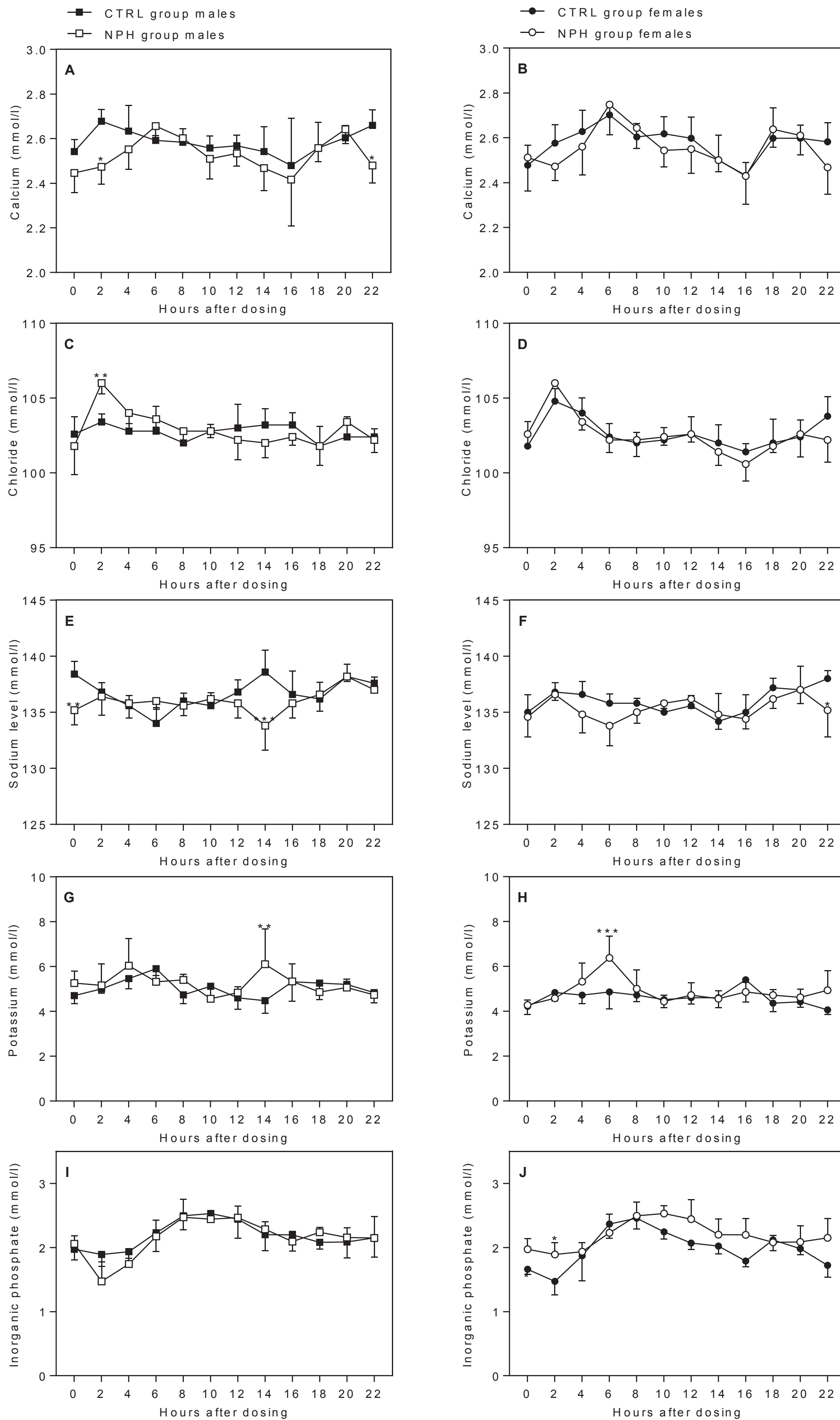

Figure 7 Effect of insulin dosing on plasma levels of electrolytes

Means±SD. For each sex: n=4-5/time-point per group. Left column: males, right column: females. Time-point zero represents a sample taken prior to s.c. dosing with NPH insulin. In case of an effect of NPH dosing a *post hoc* Sidak's multiple comparisons test was performed for each time-point separately: *p<0.05, **p<0.01, ***p<0.001 versus the CTRL group.

Discussion

The present study investigated the time-dependent effects of insulin dosing for 28 days, which should cause recurrent daily hypoglycaemia for approximately 4 h/day, on biochemical parameters. These investigations were performed to increase knowledge about the fluctuations of biochemical blood parameters caused by insulin-dosing and/or insulin-induced hypoglycaemia in non-diabetic rats in order to aid interpretation of results in non-clinical safety studies.

Insulin dosing induced lowering of plasma glucose levels from approximately 9 to <4 mmol/L with maximum effect 2 h after dosing, coinciding with peak insulin exposure. This effect tapered off within 4-6 h after dosing, similar to what has been reported by others dosing NPH insulin to diabetic rats at comparable doses (*Bellush & Reid, 1994*), and coinciding with declining insulin exposure. Insulin dosing was accompanied by increased body weights, a known effect in both non-diabetic and diabetic rats *(Willing et al., 1990; Jensen et al., 2015; Bellush & Reid, 1994*). This is in line with the fact that insulin stimulates uptake of plasma fatty acids from the blood into adipose tissue (*Gries et al., 1967*); this was also reflected by decreased triglyceride plasma levels in males for approximately 10 h after insulin dosing, although only significantly 4 h after dosing, coinciding with the duration of high insulin exposure. Insulin dosing furthermore levelled out the peaks seen 12 h apart for cholesterol levels in males. Despite the similar increase in bodyweights between NPH-Group males and females, food consumption was significantly increased only in females. Hyperphagia is a known effect of insulin dosing driven by hypoglycaemia (*May & Beaton, 1968*). The difference may be due to the fact that females are more insulin-sensitive than males *(Gustavsson et al., 2010; Gomez-Perez et al., 2008*) and, consequently, would have to increase food consumption to a higher degree than males to maintain blood glucose levels similar to insulin-dosed males, when given the same dose per kg bodyweight. An alternative explanation could be that the study was too underpowered to show a statistically significant difference with 12 levels on the time factor included in the analysis. Also, the time-dependent effect of insulin dosing approached a significant difference suggesting that a higher power would show a significant effect. Increased urea levels in the NPH-Group in both males and females most likely reflect increased hepatic gluconeogenesis, a known counter-regulatory effect to insulin dosing in rats (*Borba-Murad et al., 1999; Gazola et al., 2007*). The supply of amino acids for hepatic gluconeogenesis during periods of low glucose availability is primarily maintained by skeletal muscle tissue through *de novo* synthesis as well as proteolysis (*Snell, 1980*). In line with this, levels of the enzymes CK and LDH in females, and CK in males, were transiently increased in the NPH-Group. Increases in CK appeared 6-8 h after dosing, corresponding to a few hours after the lowest blood glucose levels were reached. This is similar to CK changes seen in rabbits with hypoglycaemia induced by human insulin (*Jiang et al., 1998; Jiang et al., 1996*), and where the increased CK was attributed to muscle tissue origin (*Jiang et al., 1998*). This suggests that increased CK levels in the present study were due to muscle tissue proteolysis supporting increased hepatic gluconeogenesis. Thus, the increased urea, CK and LDH levels in the present study probably reflect one of the vital counter-regulatory mechanisms to re-establish normoglycaemia in the rat.

In addition to the counter-regulatory measures to hypoglycaemia mentioned above, increased plasma chloride, as seen in NPH-Group males in the present study, is a known effect of insulin itself, due to direct stimulation of chloride reabsorption in the kidney *(Song et al., 2006; Kirchner, 1988*). Hypocalcaemia seen in males for 4 h after insulin dosing was most likely caused by glucagon, secreted as an immediate pancreatic counter-regulatory response to hypoglycaemia (*Burcelin & Thorens, 2001)*. Hypocalcaemia is a known effect of increased glucagon levels in rats, mediated through stimulation of calcium uptake by the bone and/or by decreasing bone resorption *(Stern & Bell, 1970; Williams et al., 1969*). Furthermore, this hypocalcaemic response has been shown to be rapid in onset (within 30 min after glucagon injection) and of short duration, lasting only a few hours (*Williams et al., 1969*), corresponding well with what was seen in the present study. Thus, in general, changes in plasma electrolytes reflected the hyperinsulinaemia, either directly or indirectly as part of the counter-regulatory response to hypoglycaemia.

When reviewing the results from present study a limitation, which should be considered, is the low n-value for each individual time-point (4-5 animals). This may have masked some changes due to low power in the *post hoc* analysis; for example, insulin dosing significantly affected plasma cholesterol levels in females and ALP levels in males, however, no significant difference was seen at any of the individual time-points. Therefore, the results should be interpreted with this in mind, as subtle changes may not

have been detected. Further investigation of changes at specific time-points may therefore be warranted if these are of interest, and the results from the present study could serve as a basis for power calculations to determine the number of animals needed to show a significant difference.

Regulatory guidelines state that toxicological evaluation of antidiabetic drugs should be performed in non-diabetic animals (*European Medicines Agency, 2001; US Food and Drug Administration, 2008*). Using non-diabetic rather than diabetic rats allows evaluation of the metabolic responses to insulin dosing, without interference from chronic hyperglycaemia. However, this also means that in contrast to diabetic animals, in which insulin lowers hyperglycaemic blood glucose levels down to normoglycaemia, as in the diabetic patient, insulin dosing to non-diabetic animals will induce hypoglycaemia. Consequently, it may be problematic to differentiate metabolic changes caused by an insulin analogue from counter-regulatory changes caused by the insulin-induced hypoglycaemia. Thus, it is important to have a detailed knowledge regarding the normal metabolic counter-regulatory changes to hypoglycaemia in non-diabetic animals when interpreting results from non-clinical safety studies. Therefore, knowledge regarding changes to biochemical blood parameters following insulin dosing in non-diabetic animals is important, as well as any sex-dependent effects as both females and males are included in these studies. Furthermore, information regarding time-dependent changes of these parameters following insulin dosing is important for optimal timing of blood sampling. To investigate this, daily insulin dosing was performed in non-diabetic rats for 28 days. On the last day of dosing, blood was sampled prior to dosing and every two hours following dosing to follow changes in blood parameters over time. Insulin dosing affected several parameters in a time-dependent manner coinciding with either peak exposure or as a delayed effect to the insulin-induced hypoglycaemia, reflecting counter-regulatory measures to regain normoglycaemia.

The effect of time of day is important to recognize when insulin dosing is performed to both non-diabetic and diabetic rats, as this may significantly affect the resulting blood glucose levels (*Haughton et al., 1999*). In fact, a study has shown that obtaining glycaemic control in diabetic rats using twice daily dosing required 50% higher insulin doses at night compared with dosing in the morning (*Haughton et al., 1999*). Insulin dosing in non-diabetic rats during the light period, when blood glucose levels are lower, might thus increase the risk of severe hypoglycaemia compared to an equivalent dose during the dark period. In the present study, dosing was performed in the morning and it could be interesting to include a group dosed in the evening to evaluate any difference in response of biochemical parameters to insulin-induced hypoglycaemia; they may be less sensitive to insulin at night due to the circadian differences in eating behaviour and thus metabolism.

Conclusions

Many of the biochemical blood parameters displayed a clear time-dependent effect following insulin dosing. Notably, effects were often not similar in males and females, emphasising the importance of including both sexes when evaluating effects on biochemical blood parameters. Additionally, as a consequence of this, optimal timing of blood sampling for the same parameter may be different in males and females.

In the NPH-Group, plasma levels of several of the biochemical parameters reflected counter-regulatory responses to the insulin-induced hypoglycaemia, typically in a time-dependent manner related to the short duration of the hypoglycaemia (≤8 h after dosing), which coincided with high insulin exposure. Counter-regulation was characterised by increased bodyweight and increased gluconeogenesis, reflected by increased ureagenesis, skeletal muscle proteolysis (increased CK and LDH), as well as hypocalcaemia. These changes were seen either coinciding with the hypoglycaemia or as a delayed effect. This highlights the need for careful consideration regarding the timing of blood sampling, i.e. focusing on which biochemical parameters are of special interest, as changes in different parameters are not necessarily present at the same time and do not always coincide with maximum insulin exposure. Results from the present study may aid the interpretation of changes in biochemical blood parameters seen in non-clinical safety studies of new drug candidates in rats.

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Declaration of Conflicting Interest

The Authors declare that there is/are not conflict(s) of interest.

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