Fasting increases serum bilirubin levels in clinically normal, healthy males but not females: a retrospective study from phase I clinical trial participants

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ABSTRACT

Aim To examine if fasting affects serum bilirubin levels in clinically healthy males and females.

Methods We used retrospective data from phase I clinical trials where blood was collected in either a fed or fasting state at screening and predosing time points and analysed for total bilirubin levels as per standard clinical procedures. Participants were clinically healthy males (n=105) or females (n=30) aged 18–48 inclusive who participated in a phase I clinical trial in 2012 or 2013.

Results We found a statistically significant increase in total serum bilirubin levels in fasting males as compared with non-fasting males. The fasting time correlated positively with increased bilirubin levels. The age of the healthy males did not correlate with their fasting bilirubin level. We found no correlation between fasting and bilirubin levels in clinically normal females.

Conclusions The recruitment and screening of volunteers for a clinical trial is a time-consuming and expensive process. This study clearly demonstrates that testing for serum bilirubin should be conducted on non-fasting male subjects. If fasting is required, then participants should not be excluded from a trial based on an elevated serum bilirubin that is deemed non-clinically significant.

INTRODUCTION

A phase I clinical trial often requires the recruitment of clinically normal males and females or ‘healthy volunteers’. Phase I clinical trials have very strict inclusion/exclusion criteria which can make the recruitment of appropriate subjects time-consuming and costly. The biochemical parameters for inclusion must be within a predefined range as documented in the clinical trial protocol. Often a single biochemical parameter that is detected outside of the predefined range is sufficient for exclusion of a willing participant from a trial. Very often, this single abnormal biochemical parameter which excludes the participant is not considered clinically significant by a medical practitioner.

Recently, we have experienced several cases where a willing participant is excluded from a trial due to elevated bilirubin levels (non-clinically significant) detected at either screening or the predose biochemical testing. These levels fall outside of the predetermined range as set by the clinical trial protocol and thus the subject is excluded despite the levels not being considered clinically significant. Even more frustrating for the willing participant is the fact that often a single biochemical parameter, in this case elevated bilirubin, is outside of the inclusion/exclusion criteria. This can lead to frustration for the willing participant and for the contract research organisation.

Commonly, participants are required to fast prior to blood collection for the medical screening. Additionally, for some studies, participants fast overnight in the clinic before baseline blood collection immediately preceding dosing with the study drug. We hypothesise that this fasting increases the levels of serum bilirubin in otherwise clinically normal participants and thus excludes willing volunteers with normal non-fasting bilirubin levels. To investigate this hypothesis, we have retrospectively examined the fed and fasting serum bilirubin levels of clinically normal males and females who participated in a phase I clinical trial.

MATERIALS AND METHODS

Study site

Q-Pharm is a specialised contract research organisation located at the Royal Brisbane and Women’s Hospital campus at Herston in Brisbane, Queensland, Australia, which undertakes a broad range of predominantly early phase clinical trials for clients in the global pharmaceutical and biotechnology industries.

Study population

All participants were clinically healthy males (n=105) or females (n=30) aged 18–48 inclusive who participated in a phase I clinical trial in 2012 or 2013. All participants successfully passed the medical screening (ie, biochemistry and haematology were within the clinically normal range) for their particular study and all were successfully dosed with the study drug. All participants had a thorough medical history recorded including no previous episodes of jaundice or any family history of liver disease. All trials were phase I trials examining the safety and tolerability of novel drugs and/or drug formulations. All participants contributed two blood samples to this study—a screening sample and a base-line (predose) sample.

Ethics

All phase I clinical trials from which we retrospectively obtained the bilirubin measurements were approved by either the QIMR Berghofer Human Research Ethics Committee or the Bellberry Human Research Ethics Committee. All trials were
conducted in accordance with the International Conference on Harmonisation Good Clinical Practice guidelines and the National Statement on Ethical Conduct in Human Research (2007) as produced by the National Health and Medical Research Council of Australia.

**Classification of bilirubin measurements as fed or fasting**

The source notes for phase I studies document the time at which the participant last consumed a meal. Therefore, we were able to determine the exact period of fasting prior to blood collection. Fasting as described in the trial source notes is no food or drink except water. For those blood samples obtained for medical screening, these fasting times are self-reported. For those blood samples obtained for baseline measurements (ie, prior to dosing), this fasting is usually during overnight confinement in the study clinic. The majority of fed measurements were done at screening and the majority of fasting measurements were performed at baseline after an overnight fast (~12 h) in the study clinic. Therefore, the accuracy of the reported fasting times is well assured. The period of time subjects were fasted prior to collection of all bilirubin measurements was calculated and graphed to enable classification into fasting and non-fasting (figure 1).

Participant bilirubin measurements were classified as fed if less than 6 h had passed since consumption of food prior to the blood being collected. Participants were classified as fasting if no food other than water had been consumed for 10–20 h prior to the blood being collected. We have excluded those fasting times that were between 6 and 10 h to better differentiate between the fed and fasting cohorts. We also removed the outlier of 21 h from further analysis due to the extreme length of this fasting period and its likely effect on clinical measurements.

**Blood collection and processing for bilirubin measurements**

The blood collection time points analysed herein were performed at two stages of the trial process: (i) medical screening and (ii) baseline blood collection prior to administration of the study drug. No blood collection performed after the administration of study drug was analysed as part of this study and all participants were classified as being clinically healthy and continued onto their particular trial and study drug dosing. All blood was sent to licensed pathology laboratories in Brisbane, Queensland, Australia, and was tested for serum bilirubin on either a DXC800 (Beckman Coulter, USA) or Abbott Architect c16000 (Abbott diagnostics, Abbott Park, Illinois, USA) platform as per standard clinical testing procedures.

**Classification of the normal range for bilirubin serum measurements**

The normal serum Bilirubin range for this study was as used by Sullivan and Nicolaides Pathology for their biochemical measurements. For females, the lower limit of normal was 3 μmol/L and the upper limit of normal (ULN) was 15 μmol/L. For males the lower limit of normal was 4 μmol/L and the upper limit of normal (ULN) was 20 μmol/L.

**Demographics of phase I trial participants analysed in this study**

All clinical trial participants self-assigned to one of four races during screening: Caucasian, Asian, Aboriginal/Torres Strait Islander or other. Participants also self-identify as being of Hispanic or Latino descent. In the five clinical trials examined for this study, approximately 85% of the participants identified as being Caucasian. The average age of the trial participants was 25.4 years (±0.44 SEM). The youngest trial participant was 18 years and the oldest was 48 years. The 25th–75th centile ranged from 22 to 27 years (figure 2).

**Statistical analysis**

The datasets were analysed using GraphPad Prism V6.00 for Windows (GraphPad Software, La Jolla California, USA). Box and whiskers graphs were used with the mean, median, 25th and 75th centile calculated for each dataset. We used the D’Agostino & Pearson omnibus normality test to test each cohort for Gaussian distribution. We used an unpaired t test to examine the significance of bilirubin levels between fed and fasting cohorts of males (Mann–Whitney) and females (Welch’s correction). We also used linear regression to examine the relationship among serum bilirubin levels, age and fasting time of the male participants.

**RESULTS**

**Serum bilirubin levels**

**Fed males**

Blood was collected from 97 clinically normal ‘fed’ male phase I trial participants at screening or predose blood collection and analysed for total serum bilirubin (table 1). The mean serum bilirubin measurement for this cohort was 10.42 μmol/L (±0.4 SEM). The 25th–75th centile ranged from 7 to 12 μmol/L. The serum bilirubin levels for this cohort were not normally distributed. The lowest measurement was 3 μmol/L and the highest reading was 21 μmol/L. Two patients (out of 97) or 2% had a serum bilirubin above the ULN; both recorded 21 μmol/L.
Fasted males
Blood was collected from 109 clinically normal ‘fasted’ male phase I trial participants at screening or predose blood collection and analysed for total serum bilirubin (table 1). The mean serum bilirubin measurement for this cohort was 14.85 μmol/L (±0.53SEM). The 25th–75th centile ranged from 11.0 to 18.5 μmol/L. The serum bilirubin levels for this cohort were not normally distributed. The lowest measurement was 4.0 μmol/L and the highest reading was 35 μmol/L. Fifteen patients (out of 109) or 13.76% recorded bilirubin measurements above the ULN. These were 21 (×2), 22 (×5), 24, 25 (×2), 27, 28, 31, 33 or 35 μmol/L.

Fed females
Blood was collected from 25 clinically normal ‘fed’ female phase I trial participants at screening or predose blood collection and analysed for total serum bilirubin (table 1). The mean serum bilirubin measurement for this cohort was 8.4 μmol/L (±0.78 SEM). The 25th–75th centile ranged from 5.0 to 11.5 μmol/L. The serum bilirubin levels for this cohort were normally distributed. The lowest measurement was 2.0 μmol/L and the highest reading was 18.0 μmol/L. One patient (out of 25) or 4% had a serum bilirubin above the ULN: 18 μmol/L.

Table 1 Serum bilirubin measurements

<table>
<thead>
<tr>
<th>Bilirubin (μmol/L)</th>
<th>Fed males</th>
<th>Fasted males</th>
<th>Fed females</th>
<th>Fasted females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=97</td>
<td>n=109</td>
<td>n=25</td>
<td>n=33</td>
</tr>
<tr>
<td>Minimum</td>
<td>5</td>
<td>7</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>25% Centile</td>
<td>7</td>
<td>11</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Median</td>
<td>10</td>
<td>14</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>75% Centile</td>
<td>12</td>
<td>18.5</td>
<td>11.5</td>
<td>12</td>
</tr>
<tr>
<td>Maximum</td>
<td>21</td>
<td>35</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>10% Centile</td>
<td>6</td>
<td>8</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>90% Centile</td>
<td>16</td>
<td>22</td>
<td>14</td>
<td>16.2</td>
</tr>
<tr>
<td>Mean</td>
<td>10.42</td>
<td>14.85</td>
<td>8.4</td>
<td>9.636</td>
</tr>
<tr>
<td>SD</td>
<td>3.856</td>
<td>5.744</td>
<td>3.905</td>
<td>3.904</td>
</tr>
<tr>
<td>SEM</td>
<td>0.3916</td>
<td>0.5502</td>
<td>0.781</td>
<td>0.6795</td>
</tr>
<tr>
<td>Lower 95% CI of</td>
<td>9.645</td>
<td>13.76</td>
<td>6.788</td>
<td>8.252</td>
</tr>
<tr>
<td>mean</td>
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<td></td>
</tr>
<tr>
<td>Upper 95% CI of</td>
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<td>10.01</td>
<td>11.02</td>
</tr>
<tr>
<td>mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Descriptive statistics for the male and female fed and fasted cohorts. Data shown include the 25th–75th centile for each cohort, the mean, minimum and maximum measurements.

Fasted females
Blood was collected from 33 clinically normal ‘fasted’ female phase I trial participants at screening or predose blood collection and analysed for total serum bilirubin (table 1). The mean serum bilirubin measurement for this cohort was 9.63 μmol/L (±0.68 SEM). The 25th–75th centile ranged from 6.0 to 12.0 μmol/L. The serum bilirubin levels for this cohort were normally distributed. The lowest measurement was 4.0 μmol/L and the highest reading was 18.0 μmol/L. Three patients (out of 33) or 9% had a serum bilirubin above the ULN: 17 μmol/L and 2×18 μmol/L.

Comparison of serum bilirubin levels between fed and fasted cohorts
To determine if fasting affected serum bilirubin levels the statistical analysis programmes in the GraphPad Prism software were used; all data points including those above the ULN were included in the analysis. Significant differences between the serum bilirubin levels in the male fed and fasted cohorts (figure 3) were found (p<0.0001). Conversely, no significant difference in the serum bilirubin levels between the female fed and fasted cohorts was identified. Fasting increased the mean serum bilirubin levels by 4.4 μmol/L in males compared with 1.2 μmol/L in females.

Fasting time increases serum bilirubin levels in clinically normal males
To further examine the effect of fasting on male bilirubin levels, we plotted the fasting times in direct comparison with the serum bilirubin levels. We then performed a linear regression and found a positive correlation between bilirubin levels and increased fasting time (figure 4). The slope of the line was 0.4313 ± 0.06733. The equation of the line was (Y=0.4313*X +9.227) where Y is serum bilirubin level and X is the hours fasted.

Age does not influence fasting bilirubin levels in clinically normal males
To examine the effect of age on male bilirubin levels, we plotted the age in direct comparison with the serum bilirubin levels. We then performed a linear regression and found no correlation between bilirubin levels and participant age (figure 5). The slope of the line was 0.09419±0.1303. The equation of the line was (Y=0.09419*X +12.61) where Y is serum bilirubin level and X is the age of the participant.

DISCUSSION
This retrospective study demonstrates a statistically significant relationship between fasting and elevated bilirubin in otherwise healthy male subjects enrolled in phase I clinical trials. These
results are consistent with previous studies including the South African study reported by Meyer and colleagues in 1995, albeit from a much smaller sample size, where they reported a rise in serum bilirubin levels in healthy males following fasting.\(^1\)

Several other studies have examined total bilirubin levels after an overnight fast. These ‘fasting’ total bilirubin levels were found to decrease following consumption of breakfast\(^2\) and/or lunch.\(^3\) These studies, while in agreement with ours, did not separate the male and female populations and were conducted in relatively small cohorts of 17 (nine males and eight females)\(^2\) and 20 (10 males and 10 females)\(^3\) subjects, respectively. We included females (25 fed and 33 fasted) in our retrospective study and found that fasting had no significant effect on serum bilirubin levels in clinically normal, healthy females.

The mechanism underlying the cause of hyperbilirubinaemia in fasted healthy males remains undetermined. Further investigation of additional confounding biochemical analytes and their response to fasting is required. We found no correlation between age and fasted bilirubin levels.

A recent review paper on clinical tests of liver metabolism by Wöreta and Alqahtani states that fasting and stress increase bilirubin.\(^4\) A study by Lopardo and colleagues in 2013 showed that fasting increases serum concentrations of Bilirubin in patients receiving atazanavir therapy for HIV.\(^5\) However, it should be noted that food affects atazanavir levels and the drug itself causes hyperbilirubinaemia.\(^5\) Lopardo and colleagues propose that an increased hepatic uptake of non-esterified fatty acids interferes with the hepatic clearance of bilirubin and thus

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**Figure 3** Comparison of serum bilirubin levels between the fed and fasted cohort of males and females (**p**<0.0001). Data are displayed as a box and whiskers plot (25th–75th centile, minimum to maximum with all data points shown). ULN, upper limit of normal; LLN, lower limit of normal.

**Figure 4** Comparison of fasting time and serum bilirubin levels in clinically normal males. A linear regression is plotted (solid line) with the 95% CI. ULN, upper level of normal; LLN, lower level of normal.
Figure 5  Comparison of age and fasting serum bilirubin levels in clinically normal males. A linear regression is plotted (solid line) with the 95% CI. ULN, upper level of normal; LLN, lower level of normal. The 21 h data point was excluded due to the extreme length of the fast.

contributes to the unconjugated hyperbilirubinaemia of fasting.  

Similar mechanisms may be at work in healthy males. 

Alternative causes of isolated elevations in bilirubin were explored. Laboratory artefact including in vitro haemolysis was excluded by adherence at all times to the recommended specimen handling and transport recommendations in addition to the inclusion of a ‘haemolysis index’ with each episode of analysis which was reported as normal. Laboratory quality assurance/quality control (QA/QC) data were reviewed and found to be within acceptable limits to ensure accuracy of the reported results and as such that there was no laboratory error to account for the reported abnormalities. In addition, total bilirubin has been shown to be suitable for clinical analysis after 24 h of storage at 4°C and room temperature in an assortment of storage tubes. 

The most common inherited disorder of bilirubin metabolism is Gilbert syndrome which is typically regarded as a benign condition characterised by recurrent episodes of jaundice occasionally triggered by a number of insults including intercurrent disease, overexertion, dehydration, menstruation and fasting. Gilbert syndrome is the result of a defect in the promoter of the gene that encodes the enzyme uridine diphosphoglucuronate-glucuronosyltransferase 1A1, which is responsible for the conjugation of bilirubin with glucuronic acid.

The hyperbilirubinaemia in patients with Gilbert syndrome is unconjugated. The current local prevalence of Gilbert syndrome is difficult to estimate; however, in different populations it has been reported to range between 4% and 16% which is not sufficient to explain the results found in this study. The prevalence is higher in males, possibly due to a relatively higher level of daily bilirubin production. The next step in this research would be to test all those who show elevated fasted bilirubin for Gilbert’s disease as definitive proof of the true incidence of Gilbert’s in the local clinical normal population.

The primary limitation of this study was that it was a retrospective analysis of data collected from a number of different phase I clinical trials. Given that the time points analysed were all prior to the receipt of any investigational product or trial drug, the subject’s participation in a clinical trial has not impacted on the results presented.

The strengths of this study include the accuracy of the documentation of the time the subjects were fasting as the majority were confined for the purpose of the clinical trial and thus being closely observed. Obviously, self-reporting of fasting is likely to be far less accurate. Further, the volume of clinical trials conducted at our facility has permitted the analysis of a large sample size to reduce the impact of any confounders. Further, given the importance of accuracy in clinical trials, strict attention was paid to standard operating procedures outlining the collection, handling and transport of pathology specimens to ensure consistency and accuracy of pathology results.

Given that all subjects analysed in this study were by definition healthy volunteers who had to have no significant medical history and a normal thorough clinical examination, the probability of intercurrent illness and concomitant medications impacting on these results is extremely low. Although there has been some reporting of this association in the literature in the past, this is by far the largest study of this type reported to date.

This study demonstrates a statistically significant association between elevated bilirubin and fasting in males. Furthermore, we have determined that this is unaffected by the age of the participant and that the length of fasting directly affects the serum bilirubin levels. These findings are important for many reasons. Healthy volunteers for clinical trials are a precious resource and so when excluded on the basis of a (not clinically significant) abnormal pathology result, a significant amount of time, effort and money is lost not to mention the impact on the conduct of the trial. Proving an association between fasting and hyperbilirubinaemia can result in the more accurate interpretation of non-clinically significant abnormal bilirubin results and provide evidence for limiting the period of fasting prior to blood collection unless fasting is absolutely necessary. This work has the potential to improve the recruitability of healthy volunteer clinical trials in addition to reducing unnecessary repetition of pathology results which can add significant expense.

Take home messages

- Fasting increases total serum bilirubin levels in clinically normal males but not females.
- We used retrospective data from fed and fasting clinically normal participants in phase I clinical trials (screening and predose blood only); thus, it is highly unlikely that other confounding diseases contributed to the elevated bilirubin levels.
- A fasting bilirubin measure that is considered non-clinically significant by a clinician should thus not be used as a clinical trial exclusion criterion if the remainder of the liver function tests are normal.
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Contributors PMG and SLE conceived the study. All authors contributed to study design and data interpretation. KJM collected the retrospective data and performed the statistical analysis. The manuscript was prepared, edited and approved by all authors.

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Competing interests None.

Ethics approval QIMR Berghofer—Human Research Ethics Committee or the Bellberry Human Research Ethics Committee.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement We have (screening) bilirubin measurements for additional participants. However, as these individuals were not successfully dosed with the study drug they were excluded from our study. Our study only included bilirubin measurements (from the screening and baseline blood samples) of those individuals who proceeded to be dosed with the study drug. These data are available by contacting the corresponding author and upon approval by Q-Pharm.

REFERENCES