Glargine blood biotransformation: 
in vitro appraisal with human insulin immunoassay

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Abstract

\textbf{Aim.} – Glargine, a long-acting insulin analogue, is metabolized in the bloodstream and in subcutaneous tissue. Glargine metabolism and its implications for diabetes therapy remain poorly understood. The aim of our study was to assess in vitro the glargine blood biotransformation and its inter-individual variability.

\textbf{Methods.} – Formation of M1 glargine metabolite in vitro was studied with Elecsys Insulin immunoassay in pools of sera and sera from patients spiked with glargine. Elecsys Insulin assay is specific of human insulin, does not recognize glargine and its M2 metabolite but does recognize its M1 metabolite.

\textbf{Results.} – Glargine incubation with serum resulted in M1 metabolite formation which was detected and characterized as an enzymatic process: metabolite kinetics were dependant on temperature, substrate concentration and serum proportion. Carboxypeptidase inhibitors and chelating agents partially inhibited the activity of the enzyme(s). Glargine biotransformation was decreased when blood was collected on EDTA tubes. After 30 min incubation of glargine (100 mU/l) in 69 sera at 37 °C, percentage of glargine converted into M1 ranged from 46% to 98% (mean 72%; S.D. 11%).

\textbf{Conclusion.} – Glargine blood biotransformation is an enzymatic process probably involving serum carboxypeptidase(s). Metabolite formation is rapid and non negligible. Inter-individual variability of glargine biotransformation is noteworthy and should be confronted to M1 metabolite bioactivity which has not been fully documented yet.

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Résumé


\textbf{Objectif.} – La glargine, analogue de l’insuline à action prolongée, est métabolisée dans la circulation sanguine et le tissu sous-cutané. Le métabolisme de la glargine et son implication dans le traitement du diabète restent incomplètement explorés. Le but de notre étude était d’évaluer \textit{in vitro} la biotransformation sanguine de la glargine et sa variabilité interindividuelle.

\textbf{Méthodes.} – La formation du métabolite M1 de la glargine a été étudiée \textit{in vitro} avec l’immunodosage Elecsys Insuline par surcharge de sérum et de pools de sérum avec de la glargine. Le dosage Elecsys Insuline est spécifique de l’insuline humaine, ne reconnaît pas la glargine et son métabolite M2 mais reconnaît son métabolite M1.

\textbf{Résultats.} – L’incubation de glargine avec du sérum résulte en la formation de métabolite M1 qui a été détectée et caractérisée comme un processus enzymatique : la cinétique est dépendante de la température, de la concentration en substrat et de la proportion de sérum. Les inhibiteurs des carboxypeptidases et les agents chélatants inhibent partiellement l’activité de la ou des enzyme(s). La biotransformation de la glargine est diminuée lorsque le sang est prélevé sur tubes EDTA. Après 30 minutes d’incubation de 100 mU/l de glargine dans 69 séums à 37 °C, le pourcentage de glargine transformée en M1 est compris entre 46 et 98% (moyenne 72% ; écart-type 11%).

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1. Introduction

Intensive diabetes therapy is performed by insulin treatment in type 1 diabetes and in insulin requiring type 2 diabetes when oral antidiabetic agents fail to provide adequate glycemic control. Strategies combining basal–bolus insulin regimens are used in an attempt to mimic physiological insulin profiles and to maintain long-term near-normoglycemia. Basal insulin is ideally a peakless, reproducible, long-lasting preparation, close to the flat interprandial physiological insulin secretion of non diabetic subjects [1]. The currently available recombinant human insulin preparations (intermediate acting NPH and long-acting ultralente) have a peak-action profile, a high variability in the day-to-day absorption and thus do not meet these criteria [2]. Genetic engineering has allowed modifications of insulin yielding to multiple modified insulins with different pharmacokinetic and/or pharmacodynamic properties. Molecules with maintained pharmacodynamic and modified pharmacokinetic profiles have been selected. Long-acting insulin analogues have been developed to more closely match the basal component of endogenous insulin secretion [3].

Glargine is one of these long-acting insulin analogues. The addition of two arginines to the insulin structure and the substitution of asparagine (A21) by glycine (Fig. 1A, B) result in a change in the net charge of the insulin molecule and shift the isoelectric point from 5.4 (human insulin) to 6.7, making glargine soluble at slightly acidic pH in preparation and less soluble at physiological pH (7.4). Insulin glargine precipitates in the subcutaneous tissue ensuring slow and steady absorption [4–6].

Compared to regular insulin preparations (NPH and ultralente), inter-individual variability has been reduced but remains an issue [7–9]. As glargine is metabolized in bloodstream and subcutaneous tissue, we suspected glargine biotransformation to be a part of this variability [10].

Serum insulin quantification in pharmacokinetic studies and in routine clinical chemistry is usually performed by immunoassay [11–14]. Specific analogue immunoassays have been developed for pharmacokinetic purposes in preclinical studies but are not commercially available for glargine. Cross-reactivity of glargine has been established in buffer with a selection of commercially available automated insulin immunoassays: only Elecsys Insulin assay seemed to have a good specificity for human insulin [15].

The aim of our study was to take advantage of specificity of Elecsys Insulin immunoassay to study glargine blood biotransformation and its inter-individual variability.

2. Materials and methods

2.1. Materials

Elecsys Insulin immunoassay (Roche Diagnostics, Meylan, France), an electrochemiluminescent automated sandwich immunoassay of human insulin, was used. Elecsys Insulin measurements were performed on an Elecsys 2010 analyzer. Elecsys Insulin uses two monoclonal anti-insulin antibodies (against human insulin) to form a sandwich complex. Absence of anti-insulin antibodies in sera and in serum pools was checked with anti-insulin RIA (Schering Cis-Bio International, GIF/Yvette, France). Insulin (Actrapid® 100 IU/ml) was obtained from Novo Nordisk (Puteaux, France) and glargine (Lantus® 100 U/ml) from Sanofi-Aventis (Paris, France). M1 and M2 (glargine metabolites) were kind gifts from Sanofi-Aventis (Professor J. Sandow, Frankfurt, Germany). Phosphate buffered saline pH 7.4, 1% bovine serum albumin (PBS–BSA), 4-(2-aminoethyl) benzenesulfonfluoride hydrochloride (AEBSF), e-aminocaproic acid (EACA), antipain hydrochloride, bestatin hydrochloride, trans-epoxysuccinyl-l-leucylamido-(4-guanidino) butane (E-64), N-ethylmaleimide (NEM), leupeptin hemisulfate, pepstatin A, 1,10-phenanthroline, potato tuber carboxypeptidase inhibitor (PTCI) and soybean trypsin inhibitor type I-S (SBTI) were obtained from Sigma-Aldrich (Lyon, France). 2-Guanidinomethylcaptosuccinic acid (GEMSA) was obtained from Calbiochem (Darmstadt, Germany). Calcium, magnesium and zinc chlorides were obtained from Merck (Darmstadt, Germany).

2.2. Samples

All the followed procedures were in accordance with the Helsinki Declaration of 1975 and the subsequent 1996 amendments. Venous blood samples were collected in glass tubes: BD Vacutainer Serum Tube or BD Vacutainer SST Advance Tube (Becton Dickinson, Le Pont de Claix, France). Blood was allowed to clot, serum was separated by centrifugation at 2000 × g for 15 min and stored at −20 °C until analysis. Plasma samples were collected in glass tubes: BD Vacutainer EDTA Tubes (K3 EDTA) and BD Vacutainer PST Tubes with an inert gel barrier lithium heparin (Becton Dickinson, Le Pont de Claix, France). Plasma was separated by centrifugation at 2000 × g for 15 min and stored at −20 °C until analysis. Serum and plasma samples were collected from patients without any insulin treatment including glargine. Hemolysis was visually assessed and hemolyzed samples were discarded.
2.3. Glargine and metabolite cross-reactivity assessment

For measurement of glargine in buffer, glargine contained in pen treatment units was successively diluted in PBS–BSA to final concentrations ranging from 10 to 200 mU/l (59–1182 pmol/l; 1 mU glargine = 5.912 pmol/l).

For determination of glargine in serum, firstly, insulin dilutions were performed in PBS–BSA to reach final concentrations ranging from 100 to 2000 mU/l. Secondly, sera were pooled; insulin level measured with Elecsys Insulin was lower than 5 mU/l, except otherwise stated. Finally, glargine in PBS–BSA was added to the serum pool (serum pool 90% volume/glargine in PBS–BSA 10% volume).

For measurement of glargine metabolites (M1 and M2) in buffer, M1 and M2 were dissolved in HCl 0.005 mol/l and successively diluted in PBS–BSA to final concentrations ranging from 50 to 1200 pmol/l for M1 and from 50 to 12,000 pmol/l for M2 (units are not defined for M1 and M2).

PBS–BSA dilutions and spiked serum pools (spiked glargine final concentration 10–200 mU/l) were then assessed by Elecsys Insulin immunoassay. Actrapid® insulin was processed as control in the same conditions. Endogenous insulin pool concentration was subtracted from results of experiments performed in serum.

2.4. Glargine biotransformation assessment

For glargine biotransformation experiments (kinetics; anticoagulant, cation and inhibitor influence; and variability), samples (serum, plasma or pooled sera) and glargine dilutions in PBS–BSA were kept frozen before utilization. All reagents and samples were allowed to stay at 4 °C before incubation.
Incubations were performed in cups for immunoassay analyzers made of clear polystyrene (Sarstedt, Nümbrecht, Germany). Glargine in PBS–BSA was added to sample (sample 90% volume/glargine 1000 mU/l in PBS–BSA 10% volume), to reach a final glargine concentration of 100 mU/l (591 pmol/l), except otherwise stated (sample A). For each sample the same procedure was performed with PBS–BSA without glargine in order to determine the endogenous insulin concentration (sample B). The incubation was stopped by cooling samples at 4 °C for 5 min. Then, insulin assay was performed for A and B samples on Elecsys 2010. Only one sample was analyzed in a run in order to get always the same delay before analysis (1 min 30 s). M1 concentration was calculated as
\[
\frac{\text{concentration of } A - \text{concentration of } B}{0.224}
\]
(cross-reactivity of M1 = 22.4%) and biotransformation was expressed in pmol/l/100 pmol/l glargine (or proportion of glargine converted into M1, %).

### 2.4.1. Glargine biotransformation kinetics

Kinetic experiments were performed with a serum pool. Glargine was incubated with various serum proportions (0%, 10%, 15%, 30% and 50%), various glargine concentrations (30, 60, 100 and 500 mU/l) and at different temperatures (4 °C, 25 °C, 37 °C and 50 °C). Measuring cell of Elecsys automate was not compatible with broad pH variation and therefore pH optimum for glargine biotransformation was not studied.

### 2.4.2. Effect of anticoagulant and meal intake on glargine biotransformation

Serum and plasmas obtained with two anticoagulants (EDTA and heparin) were simultaneously collected from three healthy subjects before and 1 hour after a meal. Glargine was incubated 30 min (linear section of serum kinetics) and 2 hours (plateau of serum kinetics) at 37 °C. The long incubation (2 hours) was chosen to verify that EDTA catalytic inhibition was lasting. Experiments on EDTA plasma were performed with and without zinc chloride (1 mmol/l).

### 2.4.3. Effect of enzyme inhibitors and cations on glargine biotransformation

Glargine and a variety of compounds (protease inhibitors and cations; see Section 2.1) were incubated with a serum pool (insulin concentration: 14.2 mU/l) for 1 hour at 37 °C. High inhibitor and cation concentrations were assessed consistently with the literature.

### 2.4.4. Inter-individual variability of glargine biotransformation

Sera from 69 hospitalized or ambulatory patients (fasting and non fasting) were incubated with glargine for 30 min at 37 °C. Incubation time was justified according to kinetic studies to assess the initial rate of metabolite formation.

### 2.5. Statistics

MedCalc 4.20 was used for statistical analysis.

### 3. Results

#### 3.1. Glargine cross-reactivity assessment

Diluted in buffer, glargine at a concentration lower or equal to 200 mU/l (1182 pmol/l) was not detectable with Elecsys Insulin assay. Table 1A shows cross-reactivities of glargine and regular insulin. After glargine incubation in the serum pool, a significant cross-reactivity was detected but results displayed were inconstant when incubation time and temperature were not controlled (data not shown). This immunoreactivity was suspected to depend on glargine biotransformation.

M2 cross-reactivity with Elecsys Insulin was negligible (Table 1B), whereas M1 cross-reactivity was estimated to be 22.4% (calculated with a M1 concentration of 200 pmol/l according to the regression equation).

#### 3.2. Glargine biotransformation kinetics

As control, the concentration of 100 mU/l (591 pmol/l) of glargine incubated during 2 hours at 37 °C in PBS–BSA was assessed using Elecsys Insulin assay. Values remained <1.39 pmol/l (analytical limit of detection). The initial rate of metabolite formation increased with the increased proportion of serum added and reached a maximum with 30% serum added to the medium (Fig. 2A). The substrate quantity (glargine concentration) was increased up to 500 mU/l (2956 pmol/l) without any saturation phenomenon: the amount of M1 metabolite formation was the same as at 37 °C but in a second phase the metabolite immunoreactivity decreased (Fig. 2C) as glargine is not stable at 50 °C (data not shown).

Table I
Cross-reactivities of glargine, M1 and M2 with Elecsys Insulin immunoassay in PBS–BSA

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<th>Cross-reactivity</th>
<th>Immunoactivity</th>
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<td>Glargine (%)</td>
<td>Regular insulin%</td>
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<td>10 mU/l</td>
<td>(59.1 pmol/l)</td>
<td>&lt;2.0</td>
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<td>30 mU/l</td>
<td>(177 pmol/l)</td>
<td>&lt;0.7</td>
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<tr>
<td>100 mU/l</td>
<td>(591 pmol/l)</td>
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<tr>
<td>200 mU/l</td>
<td>(1182 pmol/l)</td>
<td>&lt;0.1</td>
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<td>M1 (%)</td>
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<td>59.1 pmol/l</td>
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3.3. Effect of anticoagulant and meal intake on glargine biotransformation

Results of the effect of anticoagulant and meal intake on glargine biotransformation are presented in Table 2. Statistical comparisons were not performed (N = 3). Meal intake seemed to have no influence on glargine biotransformation. Glargine blood biotransformation in EDTA plasmas from fasting healthy subjects seemed lower than in sera from the same subjects. Addition of zinc chloride (1 mmol/l) to fasting EDTA plasma samples seemed to reverse partially EDTA biotransformation inhibition. Glargine blood biotransformation seemed lower in plasma (heparin) than in serum. A larger cohort of healthy subjects may be useful to confirm this finding. After 2 hours of incubation at 37 °C, differences between serum and plasma investigations remained contradictory in the three healthy subjects appealing more complete studies on this topic. In particular, subject three had a different profile of biotransformation compared with subjects 1 and 2.

3.4. Effect of enzyme inhibitors and cations on glargine biotransformation

At the studied inhibitor concentrations, only chelating agents (EDTA, 1,10-phenanthroline) and carboxypeptidase inhibitors (EACA, GEMSA, PTCI) inhibited partially glargine blood biotransformation (37 °C, 60 min incubation; Table 3). The criteria for inhibition was a relative activity lower than 90%.

3.5. Inter-individual variability of glargine biotransformation

A mean glargine proportion converted into M1 of 71.7% (S.D. 11.1%) in serum was assessed, with the results ranging from 46.0% to 97.8% (N = 69) (Fig. 3) and having a normal distribution (P = 0.69; Chi-square test). The 5th to 95th percentile interval of glargine metabolite immunoreactivity ranged from 52.5% to 87.0%.

4. Discussion

Whereas glargine cross-reactivity as determined in buffer is not detectable, attempts to determine insulin glargine cross-reactivity in serum were unsuccessful: Elecsys Insulin assay immunoreactivity displayed after incubation of glargine in serum when incubation time and temperature were not controlled was inconstant. As glargine is metabolized in vivo, we suspected a residual metabolic activity to persist in vitro. Kuerzel et al. [10] have reported glargine metabolism in subcutaneous tissue and in bloodstream. Two metabolites have been described (Fig. 1): M1 (A21-Gly-insulin) and M2 (A21-Gly-des-30B-Thr-insulin). Elecsys immunoassay does not recognize glargine and M2 but can recognize M1.

No immunoreactivity was detected when glargine was incubated without human serum, confirming the fact that biotransformation needed some serum component(s) to occur. Biotransformation was inhibited when blood was collected on EDTA (9.8% instead of 78.8% in serum); EDTA seemed to inhibit the activity of the component(s). An enzymatic mechanism could thus be hypothesized. Consequently, thereafter both incubation time and temperature were monitored. Kinetics of M1 apparition were sensitive to enzyme concentration (proportion of serum in the medium), substrate concentration (concentration of glargine in the medium) and temperature. 30% of serum in the incubation medium resulted in a maximal initial degradation rate. No limitation in substrate degradation was found up to...
supra-physiologic concentrations of 500 mU/l (2956 pmol/l). This result suggests that in vivo enzymatic degradation would not be saturated at therapeutic glargine concentrations.

Venous blood collection for glargine determination could be performed on EDTA (to limit in vitro glargine biotransformation) and frozen before analysis as biotransformation still occurs at 4 °C. Whether this procedure would be efficient to maintain glargine concentrations stable is not sure as the whole metabolism has not be fully characterized with Elecsys Insulin (M2 has not been measured in this study).

As a complete inhibitor of these enzyme(s) could not be found to accurately monitor time of incubation, no enzymatic constant could be calculated (biotransformation may still occur during M1 metabolite Elecsys assessment). Complete inhibition would allow a precise determination of the values of the catalytic constants.

Human insulin has two main antigenic determinants: epitopes have been identified on the A-chain (residues A4, A8–A10) and on the B-chain terminus (residues B27–B30) [16, 17]. Insulin analogues can show various degrees of cross-reactivity with human insulin immunoassays depending on the selection of the monoclonal antibody used in the commercial reagents [18]. Elecsys Insulin uses two monoclonal antibodies: MAK-Bi (R1) which recognizes the A7–A10 region.
and Fab-Ru (R2) which recognizes the C-terminal part of insulin B-chain [19]. Thus, glargine (different from insulin on its B-chain C-terminal part) was not recognized by Elecsys Insulin (as stated in buffer medium). In buffer, Elecsys Insulin fails to recognize insulin analogues (lispro, aspart and glargine) [15]. All these analogues are modified at the C-terminal end of the B-chain. Monoclonal antibody in R2 reagent recognizes the C-terminal part of insulin B-chain. Thus, this antibody may not recognize analogues and B-chain C-terminus modified metabolites. Among glargine metabolites, M1 is the only one to be strictly identical to insulin B-chain in its C-terminal part (Fig. 1A, C). Thus, M1 is recognized by Elecsys Insulin immunoassay. No data are available about the exact scheme of glargine metabolism: successive degradation (glargine \(ightarrow\) A21-Gly-B31-Arg-insulin \(\rightarrow\) M1 \(\rightarrow\) M2) or independent metabolic pathways. Better understanding of this scheme is important for sample handling to avoid enzymatic process in vitro before analysis in glargine studies.

Among enzymes processing insulin from proinsulin, proproteases (PC2 and PC1/3), carboxypeptidases or similar enzymes are candidates for such an activity [20,21]. PC2 and PC1/3 are serine proteases. Serine protease inhibitors tested in our experiments failed to inhibit glargine blood biotransformation. Moreover, glargine structure may not be a suitable substrate for these enzymes. The inhibition of catabolic reactions by EDTA and 1,10-phenanthroline suggests the participation of a zinc metallopeptase to the glargine biotransformation. Moreover, EDTA inhibition was reversed by Zn\(^{2+}\). Metalloproteinase catalyze the hydrolysis of peptide bonds at the C-terminus of proteins and peptides [22].

Among metalloproteinases, carboxypeptidase H (EC 3.4.17.10, also known as carboxypeptidase E, CPH, CPE), a carboxypeptidase B-like enzyme, extracted from insulinoma tissue is able to remove basic amino-acids from the C-terminus of diarginyl-insulin (insulin with two arginine residues at positions B31 and B32), intermediate product in the process of proinsulin to insulin. But pH optimum of CPH is in the range of 5–6 and this enzyme has little activity above pH 6.5 [23]. Moreover, CPH is mainly localized in endocrine (pancreas) and neuroendocrine tissues. In plasma, 2 metalloproteinases are present: carboxypeptidase U (EC 3.4.17.20, CPU, also known as carboxypeptidase R, activated thrombin-activatable fibrinolysis inhibitor, TAFIa) and carboxypeptidase N (EC 3.4.17.3, CPN, also known as lysine carboxypeptidase, anaphylatoxin inactivator, kininase I) [24, 25]. Both carboxypeptidases can remove C-terminal arginine or lysine from proteins and peptides. CPU has a preference for terminal arginine over lysine whereas CPN is more specific for lysine [26].

Plasma procarboxypeptidase U, CPU proenzyme, is present in plasma at a concentration of 4–10 \(\mu\)g/ml and has recently been renamed thrombin-activatable fibrinolysis inhibitor (TAFI) in relation with its role in coagulation and fibrinolysis [25]. TAFI is proteolytically activated during blood coagulation by the thrombin–thrombomodulin complex. Thus, TAFI activity is not detectable in human plasma, but appears after blood coagulation. Activated TAFI (TAFIa, CPU) enzymatic activity is highly sensitive to temperature (TAFIa half-life: 10 min at 37 °C, 45 min at 30 °C, several hours at 22 °C and stable at 0 °C) [25]. Thus, TAFIa participation to in vitro glargine blood biotransformation is possible in serum.

CPN is secreted by the liver in the bloodstream where it is present at high concentrations (30 \(\mu\)g/ml). CPN is constitutively active, stable in plasma and plays a major regulatory role as inactivator of a large variety of potent peptides and proteins such as kinins and anaphylatoxins [22]. CPN is a good candidate for its participation to the enzymatic glargine degradation process.

However, carboxypeptidase inhibitors (EACA, GEMSA, PTCI) failed to completely inhibit glargine biotransformation in experimental conditions suggesting a high activity of these enzymes in serum or the participation of other enzyme(s). Healthy subject 3 (Table 2) displayed a different pattern of results than the two other healthy subjects depending on blood collection (with or without heparin). This result is consistent with the involvement of several enzymes in glargine biotransformation. Serum or plasma assessment of glargine biotransformation may be a critical issue.

Inter-individual variability has been reported for glargine: in 18% of type 1 diabetes patients, one injection per day of glargine is not sufficient to maintain normoglycemia [27]. The pharmacological bioactivity of glargine metabolites has been suggested by Kuerzel et al. [10] but is not yet clearly demonstrated. This issue needs to be clarified. Differences in enzymatic activity among diabetic population could explain a part of inter-individual variability. In vitro serum glargine biotransformation into M1 ranges from 46% to 98% of glargine spiked to the tube after a 30 min incubation at 37 °C. If TAFIa participation is confirmed, handling of serum samples may be a critical issue for variability (TAFIa half-life is several hours at 22 °C). In the “variability study”, time between blood collection and centrifugation was not controlled whereas it has been controlled for the “healthy subject study”. In vitro enzyme activity and its inter-individual variability are quite important and do not represent the whole in vivo metabolism as glargine is also metabolized in subcutaneous tissues and M2 production occurs. In vivo, glargine is not soluble in the subcutaneous injection site and may be therefore protected from degradation in metabolites; furthermore, a significant proportion of glargine may be cleared from bloodstream before metabolism can occur.

Kurtzhals et al. [28] have compared in vitro the IGF-1 receptor binding properties and mitogenic potencies of insulin analogs including glargine and M1. Using purified human receptors, relative IGF-1 receptor affinities were 641% and 42% compared to insulin (100%) for glargine and M1, respectively. Using human osteosarcoma cells, relative mitogenic potencies were 783% and 34% compared to insulin (100%) for glargine and M1, respectively. Glargine mitogenic potency has not been confirmed in other cellular models [29–31]. However, glargine failed to have carcinogenic properties in an animal study [32]. As M1 has a very low mitogenic...
potency and probably represents a significant proportion of glargine in vivo, glargine metabolism may explain discrepancies with mitogenic properties comparing in vitro and in vivo data.

The population of the inter-individual variability of glargine biotransformation was composed of ambulatory and hospitalized non diabetic patients. These preliminary data have to be confirmed in type 1 and type 2 diabetic populations without any critical illness. Bioactivity of metabolites has to be assessed before being able to correlate clinical observations of glargine inter-individual variability and its metabolism. These preliminary results urge further studies to characterize glargine metabolism and to correlate these data with clinical observations. As glargine metabolism is still poorly understood, studies are needed in diabetic patients and particular clinical situations including enzymatic inhibitors and heparin treatments.

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