



ORIGINAL ARTICLE

Correlation between FIX genotype and pharmacokinetics of Nonacog alpha according to a multicentre Italian study

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Introduction: Pharmacokinetic (PK) studies on recombinant FIX concentrate, Nonacog alpha, were conducted with different sampling time designs which gave rise to not complete and homogenous outcomes. In addition, patient's FIX genotype/PK relationship has never been investigated. **Aim:** Investigate how different sampling times may affect PK parameters and try to find a FIX genotype/PK relationship. **Patients and Methods:** A cohort pharmacokinetic, Nonacog Alpha single-dose, open-label, non-comparative study was conducted in eight Comprehensive Care Haemophilia Centres in Italy. Seventeen previously treated moderate or severe haemophilia B patients were enrolled. Factors IX:C one-stage clotting assay, FIX genotype and PK analysis were centralized. **Results:** The evaluation of PK outcomes showed a quite long half-life, smaller clearance and volume of distribution of Nonacog Alpha in comparison with the results from previously reported studies, where blood sampling was stopped too early. The relationship between PK outcomes and FIX genotype showed that small deletions displayed the higher clearance and shorter half-life, the nonsense mutations (the lower and the longer respectively), and missense mutations were in between. **Conclusions:** It is evident that area under the curve (AUC) and other PK parameters depend from the sampling time design. In order to have a complete evaluation of clotting factors *in vivo* decay, blood samples must be collected until the baseline factor concentration has been achieved again. Due to the relationship between FIX genotype and clearance, tailored prophylaxis of HB patients could be partially predicted by genotyping.

Keywords: DNA Recombinant proteins, factor IX, genotype, haemophilia B, pharmacokinetics

Introduction

Very few well-designed studies have been conducted to achieve complete information about the PK of rDNA derived factor IX concentrate, Nonacog Alpha,

in haemophilia B patients. In some studies, the sampling times or PK analysis were not adequate, sometimes limited to 48th hour, sometimes extended to 72nd hour. In addition, the FIX genotype of patients was never reported.

Nonacog Alpha (BeneFix™, Pfizer Inc, New York, NY, USA), recombinant coagulation factor IX, is a 415-amino acid glycoprotein (approximately 55k.Da) produced in Chinese hamster ovary (CHO) cells. It belongs to the so called 'Third generation recombinant concentrates' because the medium where the cells grow and the final formulation do not contain

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any animal or human protein. After two steps of purification by ion exchange and affinity chromatography, Nonacog alpha is submitted to nanofiltration (20 nm pores) in order to remove any pathogens, eventually present in the cell culture. The first article [1] published in 1997 was a report of the regulatory bioequivalence study conducted in a small cohort of patients ($n = 11$) comparing Nonacog alpha against a monoclonal plasma-derived FIX (pdFIX) concentrate. Only *in vivo* recovery (IVR), half-life (HL) and mean residence time (MRT) were reported. Another crossover study confirmed the low IVR of Benefix with respect to pdFIX even though HL was similar in both products [2]. A well-designed study on a large cohort of patients ($n = 56$) conducted at the switch from pdFIX concentrate to Nonacog alpha reported again similar outcomes, but again limited to IVR and HL [3]. The raw data of another previously published study [4] were afterwards analysed by S. Bjorkman in order to determine the standard pharmacokinetic parameters according to a one- and two-open compartment model: for the first time, also clearance (Cl), volume of distribution at steady state (Vdss), and terminal half-life ($t_{1/2}$) were published further than IVR and MRT [5]. A new double-blind, randomized, pharmacokinetic crossover study involved the new reformulated Nonacog alpha in comparison with the original Nonacog Alpha [6]. Analysis of AUC, both percentage as well as incremental IVR, and terminal half-life did not show any difference between the two formulations. Most of these studies have been conducted with different and sometimes inadequate study designs. Only the analysis performed by S. Bjorkman [5] provided a complete report of all the PK parameters needed for a good evaluation of Nonacog alpha. For these reasons, we planned to conduct in Italy a multicentre PK study designed according to the ISTH recommendations [7,8], taking into account also the FIX genotype of each patient.

The primary objective of the study was to investigate the variability in all PK parameters (*in vivo* recovery, AUC, clearance, MRT, elimination rate constant, volume of distribution, terminal half-life of Nonacog alpha and the correlation between FIX genotype and PK outcomes, in severe and moderate haemophilia B previously treated patients. The secondary objective was to evaluate different methods of PK analysis.

Patients and methods

Single-dose PK was performed in eight Comprehensive Care Haemophilia Centres (HCC) of Italy. Seventeen moderate and severe haemophilia B patients have been enrolled in the study. PK study was performed according to the ISTH recommendations: after a wash out of

at least 7 days, a single dose, $47.70 \pm 4.13 \text{ IU kg}^{-1}$ b.w., of Nonacog alpha was injected in less than 10 min and blood samples were collected just before the infusion (baseline FIX concentration) and 0.25, 1, 3, 9, 24, 48, and 72 h after the end of infusion. Three aliquots of platelet poor citrated plasma of each sample were snap frozen and stored at -80°C . The samples were shipped within 30 days, in dry ice to the central laboratory located in the Haemophilia Centre of Florence. FIX:C was assayed by means of one-stage clotting method, performed by means of coagulometer Behring Coagulation Time (BCT) of Siemens Healthcare Diagnostics using three double dilutions of each sample compared with a reference curve of standard human plasma (Siemens Healthcare GmbH, Erlangen, Germany). FIX inhibitor was assayed on the baseline sample, according to Nijmegen method. FIX genotype was performed on whole blood samples at Haemophilia Centre of Castelfranco Veneto by means of Conformation Sensitive Gel Electrophoresis (CSGE) [9], a rapid non-radioactive heteroduplex-based method, followed by sequencing of the purified gene fragment suspected to contain the molecular defect. Comparison with the wild type is performed using the BLAST software, which is developed and maintained by a group at the National Centre for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov).

The study was approved by the Ethical Committee of Coordinator Centre and by each of other HCC (EudraCT n. 2008-006465-85). It was planned to enrol about 50 HB patients, but unfortunately, many patients denied undergoing such demanding protocol. We decided to stop the study after 5 years when only 17 patients agreed to participate to this study.

Pharmacokinetic analysis

PK analysis was conducted by means of Phoenix WinNonlin 6.3 software (Certara USA Inc., Princeton, NJ, USA). Non-compartmental analysis (NCA), one- and two-compartment methods (OCM and TCM) were used to evaluate the behaviour of infused Nonacog alpha and to compare these outcomes with FIX genotype of patients. The baseline FIX:C concentration has been subtracted from each postinfusion concentration of PK.

The parameters reported on Table 1 (Elimination rate constant, IVR, Terminal Half-life, AUC, Clearance, MRT, and Volume of distributions) have been calculated by means of NCA. In addition, OCM and TCM provided K1-0, i.e. the elimination rate constant from central compartment and the corresponding half-life (K1-0_HL). TCM also provided the alpha elimination rate constant associated with the distribution phase and beta elimination rate constant associated with the elimination phase and their respective half-lives. In order to evaluate which compartmental model

Table 1. Non-Compartment and Two-Compartment Analysis of Nonacog alpha pharmacokinetics.

Non-compartment analysis	Unit	Mean	1 SD
Incremental <i>In Vivo</i> Recovery	IU dL ⁻¹ IU ⁻¹ kg ⁻¹	1.21	0.31
Squared R adjusted	R ²	0.977535	0.02166
Elimination constant rate	1 h ⁻¹	0.033224	0.030618
Terminal Half-life	Hours	29.81	13.22987
Peak	U dL ⁻¹	57.52	14.9
Trough	U dL ⁻¹	7.71	4.15
AUC observed, last point	U*h dL ⁻¹	1442.58	426.33
AUC observed, infinity	U*h dL ⁻¹	1835.78	672.12
Clearance	mL h ⁻¹	228.30	147.0
Clearance normalized by BW	mL h ⁻¹ kg ⁻¹	3.01	1.35
MRT observed, last point	Hours	23.76	3.51
MRT observed, infinity	Hours	42.12	15.07
Half-life according to MRT	Hours	29.19	10.44
Volume of distribution (Vd) according to terminal half-life, normalized by BW	mL kg ⁻¹	110.46	30.78
Volume of distribution (Vss) at steady state, normalized by BW	mL kg ⁻¹	111.1	22.66
Two-compartment model			
AUC	U*h dL ⁻¹	1950.15	649.50
K10 Half-life	Hours	22.30	8.04
Alpha, Distribution Half-life	Hours	4.50	4.43
Beta, Elimination Half-life	Hours	38.35	23.10
Cmax	IU dL ⁻¹	63.52	21.11
Clearance	mL h ⁻¹	196	69
Clearance normalized by BW	mL h ⁻¹ kg ⁻¹	2.69	0.89
MRT	Hours	49.58	23.71
Vss normalized by BW	mL kg ⁻¹	121.59	56.09

fits better the PK data, we calculated the sum of squared residuals (SSR) according to OCM and TCM. We assumed that the model best fitting the observed data was that with the smaller SSR. Incremental recovery was calculated as ratio between the FIX:C peak (IU dL⁻¹) and dose kg⁻¹ (IU kg⁻¹), according to Prowse [10].

Results

The means and 1 SD of each PK outcomes are reported on Tables 1 and 2. The average age of patients was 34.5 years (range: 15.4–60.6 years), and weight average was 74.0 kg (range: 50.0–110.0 kg). The average FIX:C at baseline was 1.2 IU dL⁻¹ (range: 0.1–5.1 IU dL⁻¹) and FIX inhibitor was 0.04 BU mL⁻¹ (range: 0.00–0.10 BU mL⁻¹). Table 1 reports the outcomes of PK analysis, as far as NCA and TCM methods are concerned. A very good correlation between times and rFIX concentration has been shown by the high values of adjusted R², according to NCA. This is the reason to evaluate the elimination constant rate and terminal half-life by NCA; the linear regression analysis was performed by WinNonlin on about all postinfusion points (generally six out of seven). The average terminal half-life resulted near 30 h, but in seven out of 17 patients was longer than

30 h. In addition, OCM and TCM confirmed these values, both MRT as well as HL, even though the HL according to the elimination rate from the central plasma compartment (K1-0 HL) of both models were lower, 20.64 and 22.30 h respectively. Nevertheless, the beta elimination HL by TCM (38.35 h) was very similar to MRT_{inf} of NCA (42.12 h). Very long MRT resulted also by TCM, i.e. 49.58 h. The trough level of rFIX at 72 h was still high; mean 7.7 IU dL⁻¹ (3–15 IU dL⁻¹). This observation has been confirmed by the high percentage (18.6%) of extrapolated AUC to infinity. The clearance and volume of distribution at steady state of Nanacog alpha normalized by patients' body weight resulted 3.09 mL h⁻¹ kg⁻¹ and 111.1 mL kg⁻¹ respectively. A paired t Student test comparing the outcomes of OCM and TCM showed statistically significant difference ($P < 0.001$) between the two models, as far as AUC, AUMC, Cmax, Cl, MRT and Vss are concerned (data not shown). The statistically significant smaller SSR achieved by TCM with respect to OCM (45.26 vs. 143.23, $P < 0.001$) shows that TCM fits the Nonacog alpha PK data better than OCM. The range of incremental IVR was 0.89–2.23 IU dL⁻¹ IU⁻¹ kg⁻¹; the mean being 1.22 IU⁻¹ dL⁻¹ IU⁻¹ kg⁻¹ (1.14 IU⁻¹ dL⁻¹ IU⁻¹ kg⁻¹, short of the highest value).

All genotypes observed in our cohort were classified into three groups: 1 – Small base deletion (SBD, $n = 2$); 2 – Missense mutation (MM, $n = 10$); 3 – Nonsense mutation (NM, $n = 3$). Due to the small size of these groups, we reported in Table 2 only the ranges of each PK outcome observed. According to NCA, both patients of the first group displayed the shortest terminal half-life or MRT, and the higher clearance with respect to the all other patients. On the contrary, the longest terminal half-life and the smaller clearance was observed in the three patients of group 3, even though a partial overlap with patients of group 2 was reported. Cmax, IVR, AUC, and Vss were randomly distributed among the three groups. Even though TCM resulted more accurate than OCM because of its definitively reduced SSR (data not shown), beta HL, clearance and MRT according to TCM did show less significant clustering of outcomes within the genotypes as observed by NCA. A summary of these data are reported in Fig. 1. Among the outcomes of TCM, a good correlation has been observed between MRT and beta HL ($R^2 = 0.97$, $P < 0.01$), as well as between terminal half-life and MRT evaluated by NCA ($R^2 = 0.61$, $P < 0.02$). Both Clearance from NCA and TCM were well correlated ($R^2 = 0.80$, $P < 0.01$). On the contrary, no correlations have been found in our study between MRT by NCA and by TCM, neither between IVR and Vss nor between terminal half-life by NCA and beta HL by TCM.

Discussion

The figure of IVR is a bit higher than that, 0.78 ± 0.24 or $0.86 \pm 0.21 \text{ IU}^{-1} \text{ dL}^{-1} \text{ IU}^{-1} \text{ kg}^{-1}$, reported by first studies [1,2], confirmed also after the Nonacog alpha reformulation of diluent ($0.73 \pm 0.20 \text{ IU}^{-1} \text{ dL}^{-1} \text{ IU}^{-1} \text{ kg}^{-1}$) [6]. Very recently, higher values of IVR of Nonacog alpha, $0.93 \pm 0.31 \text{ IU}^{-1} \text{ dL}^{-1} \text{ IU}^{-1} \text{ kg}^{-1}$, have been reported by L.A. Valentino [11] and a very similar figure to our IVR by T. Andreeva at ASH Annual Meeting 2015: $1.24 \pm 0.32 \text{ IU}^{-1} \text{ dL}^{-1} \text{ IU}^{-1} \text{ kg}^{-1}$ [12]. These changes of Nonacog alpha concentrate IVR during the last years might reflect some changes in the manufacturing procedures or of the labelled potency.

An accurate and comprehensive PK analysis according to three methods (NCA, OCM and TCM) was able to characterize the Nonacog alpha *in vivo* behaviour better than in previous studies. The timing of

sample collection is crucial to achieve outcomes that are more reliable. Even though the ISTH recommendation stated for PK of FIX concentrate that the last point could be the 72 h, more than 18% of AUC was lost in this study, due to very high trough of decay curve. Unfortunately, none PK studies respected the golden rule of pharmacokinetics that sample collection must be continued up to the baseline is again achieved. On the other hand, it is difficult to forecast *a priori* the time of next achieved baseline. Also in our study, the range of trough was high. In all recent published comparative studies between new long-acting rFIX and Nonacog alpha, the sampling time of the comparators, pdFIX and Nonacog alpha, has been stopped too early, generally at 48 h after the end of infusion [13–15]. This is the reason why lower values of AUC, higher clearance and shorter half-life have been reported with respect to the outcomes of our study. On the contrary, when the sampling time was

Table 2. PK outcomes by NCA and TCM according to HB patients' genotype.

	Non-compartment analysis						Two-compartment method		
	Terminal HL Hours	Cmax IU dL ⁻¹	AUC U*h dl ⁻¹	MRT Hours	Cl mL h ⁻¹ kg ⁻¹	Vss mL kg ⁻¹	Beta_HL Hours	CL mL h ⁻¹ kg ⁻¹	MRT Hours
Small base deletion <i>n</i> = 2									
Min	5.02	47.7	673	15.57	4.17	76	19.04	2.64	27.14
Max	9.69	59.3	1227	18.08	6.73	106	30.14	3.22	41.72
Missense mutation <i>n</i> = 10									
Min	14.17	41.2	812	17.69	1.51	75	20.01	1.26	26.34
Max	42.20	106.3	2393	27.27	5.22	134	105.65	3.25	108.85
Nonsense mutation <i>n</i> = 3									
Min	33.84	52.0	1420	25.17	1.63	98	34.60	1.07	46.73
Max	57.45	64.2	2060	26.76	2.76	162	78.89	2.08	95.92

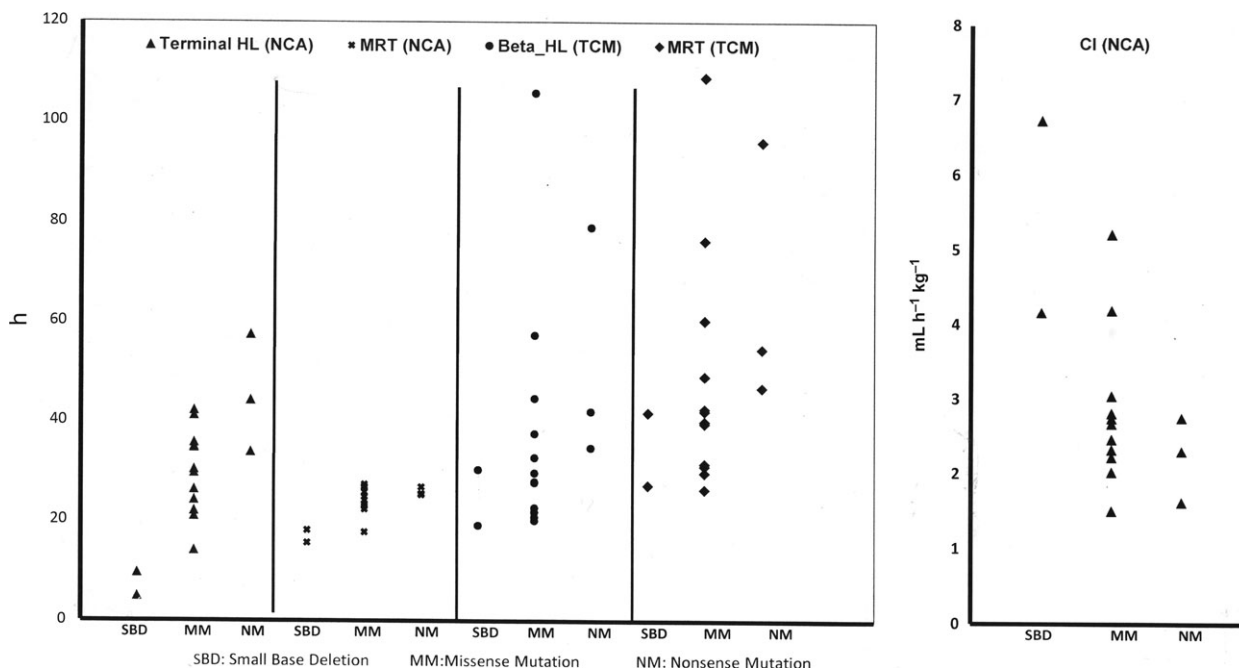


Fig. 1. Terminal half-life, MRT and clearance according to NCA; MRT and beta half-life according to TCM.

prolonged up to 72 h [16–18], the PK outcomes resulted very similar to ours. The average values of all clearances observed in our study resulted 50% less than those reported in previous comparative studies of N9-GP or rFIX-FP [14,15] because of different AUC. The clearance observed in this study is similar to that of rFIX-Fc [17]. In addition, the V_{ss} was quite small compared with the large V_{ss} of rFIX-Fc, whose mean value was 250 mL kg^{-1} [19]. The large V_{ss} of rFIX-Fc is probably due to its uptake by Fc receptor bearing cells.

Even though the size of this study is small, due to the difficulties to enrol HB patients in such a demanding protocol, the relationship between genotype and PK outcomes resulted quite interesting. Patients with SBD and with NM showed an opposite behaviour as far as terminal half-life, clearance, MRT and beta half-life were concerned. Outcomes of NCA seems to be able to better discriminate differences among the three groups of genotype. NCA is confirmed to be the most robust and reliable method of analysis of PK data because it is not affected by variance and errors of fitting procedure of all compartment methods. Model-independent method is more reliable than compartment methods both for FVIII and FIX concentrates, as our working group stated many years ago [20,21]. OCM resulted definitely less accurate than TCM because of its higher SSR. TCM seems to fit better the PK data of rFIX, as also recently reported [22]. We would like to recommend that the choice of the best fitting compartment method should be based on SSR.

Conclusion

The analysis of single-dose PK multicentre study showed different results from those previously

published because of different sampling times. Even though the last sample was collected 72 h after the end of infusion, 18% of Nonacog Alpha AUC was lost. Clearance resulted about 50% less of that reported in studies where the sampling was stopped at 48 h. Higher values of terminal half-life, MRT and beta half-life have been observed by NCA or TCM. TCM fitted very well the PK data as shown by the very low SSR. Two patients with SBD displayed the shortest terminal half-life and the higher clearance than those with MM or NM. Due to the relationship between FIX genotype and clearance, tailored prophylaxis of HB patients can be partially predicted by genotyping.

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Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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Appendix

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