Utility of a high VWF:FVIII ratio in preventing FVIII accumulation: a study in VWF-deficient mice

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Treatment of von Willebrand disease typically requires multiple infusions of von Willebrand factor (VWF)/factor VIII (FVIII) concentrate. Accumulation of FVIII is a clinical concern due to potential risk for thromboembolism. This study sought to determine whether VWF/FVIII concentrate of high VWF:FVIII ratio can prevent FVIII accumulation. VWF-deficient knockout mice received four 150 IU/kg VWF:ristocetin cofactor (RCo) infusions at 3-h intervals, with VWF/FVIII concentrates of a high (Haemate P/Humate-P) or low (Wilate) VWF:FVIII ratio. After each infusion, trough FVIII and VWF levels in plasma were determined. Separately, pharmacokinetic analysis was performed after single 250-IU/kg VWF:RCo infusions of each concentrate. Over the course of the four infusions, trough FVIII increased significantly in the group receiving Wilate ($P < 0.001$), but not Haemate P/Humate P ($P = 0.058$). After the first infusion, mean trough FVIII level in the Wilate group (31.7 IU/dl) was greater by 82% ($P = 0.017$) than that in the Haemate P/Humate P group (17.4 IU/dl). After the final infusion, mean trough FVIII of animals receiving Wilate (55.1 IU/dl) continued to exceed that of Haemate P/Humate P recipients (30.2 IU/dl) significantly ($P < 0.001$). Trough VWF levels were similar in the two groups. The VWF pharmacokinetics of the two concentrates coincided closely; however, the FVIII peak concentration and area under the curve were approximately twice as great in the mice treated with Wilate. In a murine model of severe von Willebrand disease, a VWF/FVIII concentrate with a high VWF:FVIII ratio prevented persistent exposure to elevated trough FVIII levels. *Blood Coagul Fibrinolysis* **26:515–521**

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Introduction

von Willebrand disease (VWD) – a common but heterogeneous inherited bleeding disorder – is caused by quantitative or qualitative defects in von Willebrand factor (VWF). VWD is classified into three different primary types, which vary in severity. The mainstay for treatment of patients with more severe VWD is plasma-derived concentrates containing VWF and factor VIII (FVIII) [1–3]. These include patients with type 3 VWD, many with variants of type 2 VWD and those with the most common type of VWD (type 1), who either are not candidates for treatment with the synthetic vasopressin analog desmopressin or are not responders to that agent. Pharmacokinetic analysis is used to tailor treatment in VWD patients and establish bioequivalence of new and older products, and both VWF and FVIII levels are monitored.

von Willebrand factor and FVIII circulate together in plasma, and a major role of VWF is to protect FVIII from inactivation [4]. VWD patients can have a secondary coagulation deficiency of FVIII, because the half-life of FVIII can be radically shortened in patients with VWD. In type 3 VWD patients, who have virtually a complete lack of VWF, the observed half-life of FVIII concentrate infused alone was reported to be only 2.5 h [5]. By providing both VWF and FVIII, concentrates immediately correct the coagulation deficiency and can prevent or stop bleeding. However, VWD patients also produce endogenous FVIII, which becomes stabilized by the administered VWF. Consequently, repeated infusions of concentrate given for severe bleeding episodes or surgical prophylaxis, combined with endogenously synthesized FVIII, may result in elevated plasma levels of FVIII [6–8].

In non-VWD patients with a history of venous thromboembolism, persistently elevated levels of FVIII have been associated with increased risk of both prior and recurrent venous thrombosis [9]. While the reported incidence of thrombotic events in patients receiving VWF/FVIII concentrates has been very low [10–13], the possibility that FVIII elevation may increase thrombotic risk in VWD is a recognized concern, and maintenance of FVIII levels no higher than 250–300 IU/dl during VWF/FVIII therapy has been recommended [3,14].

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A number of replacement concentrates are commercially available, and the ratios of VWF:ristocetin cofactor (RCo) to FVIII coagulant activity (FVIII:C) range from 0.55 to 2.4 [15,16]. We previously evaluated in normal rabbits the effects of repeated infusions with two VWF/FVIII concentrates differing in VWF:FVIII ratio on attained FVIII trough levels and pharmacokinetic parameters [17]. The results suggested that exposure to elevated FVIII levels can be reduced through use of VWF/FVIII concentrates with higher VWF:FVIII ratios. A limitation of that study was the endogenous production of VWF in normal rabbits.

Mice deficient in VWF (VWF−/−) have been established as a suitable model of human type 3 VWD [18,19]. In this study, trough FVIII and VWF levels were determined in VWF-deficient mice after repeated infusions of two VWF/FVIII concentrates, one with a high VWF:FVIII ratio of 2.4 (Haemate P/Humate P) and the other with a low ratio of 0.9 (Wilate). Pharmacokinetic parameters were also characterized after single infusions.

Methods

Animals

Male and female VWF-deficient mice [18] more than 8 weeks old, weighing 22–39 g, were bred and supplied by Charles River Laboratories (Erkrath, Germany). VWF:antigen (VWF:Ag) is undetectable in VWF-deficient mice [18]. Due to the lack of stabilizing VWF, FVIII activity in these mice is decreased to less than 20% of the wild-type levels [18]. The animals were housed in polycarbonate cages with a bedding of wood shavings (Braun, Battenberg, Germany) at 20–24°C and 40–50% relative humidity under a 12-h/12-h light–darkness cycle. They were fed standard mouse diet (Ssniff-Versuchsdiäten, Soest, Germany), and tap water was provided *ad libitum*. The conduct of the study was in compliance with the European Convention on Animal Care and under the approval of the organizational Ethics Committee.

Endpoints

The primary study endpoint was FVIII and VWF trough level following repeated infusions of VWF/FVIII concentrates. The secondary endpoint was the pharmacokinetics of FVIII and VWF after a single VWF/FVIII concentrate infusion.

Treatment

Mice received equal intravenous dosages of Haemate P/Humate P (CSL Behring GmbH, Marburg, Germany) or Wilate (Octapharma AG, Lachen, Switzerland) by bolus injection into the lateral tail vein. The ratio of VWF:RCo to FVIII:C averages 2.4 in Haemate P/Humate P [20] and 0.9 in Wilate [21].

For trough assessment, five female VWF-deficient mice per group received one, two, three or four 150-IU/kg VWF:RCo infusions of Haemate P/Humate P or Wilate at 3-h intervals for up to 9 h. The 3-h dosing interval was selected on the basis of the relatively short reported half-life of VWF in VWF-deficient mice (2.2–2.9 h [22]) compared with type 3 VWD patients (12.4–17.1 h [23]), necessitating more frequent doses than in the clinical setting to ensure sustained elevation of VWF levels above baseline. For pharmacokinetic evaluation, 12 male VWF-deficient mice per group received single 250-IU/kg VWF:RCo Haemate P/Humate P or Wilate infusions.

Blood samples

From the groups of mice receiving one, two, three or four VWF/FVIII infusions blood samples were drawn under deep anesthesia at 3, 6, 9 and 12 h, respectively, following the first infusion, so that only a single blood sample was collected from each individual animal for trough level determinations. For pharmacokinetic analysis, two blood samples were drawn from each individual mouse after VWF/FVIII concentrate infusion. Sampling time points after infusion for pharmacokinetic analysis were 5, 30 and 60 min and 2, 4, 6, 8 and 16 h, with three samples being secured at each time point in each group. All blood samples were citrated by mixing with sodium citrate solution 3.13% in proportions of 1 : 9 and stored at approximately −70°C until measurement.

Assays

ELISAs were used to measure plasma concentration of human VWF:Ag with antibodies from Dako Denmark A/S (Glostrup, Denmark) and FVIII:Ag with antibodies from Cedarlane Laboratories Limited (Hornby, Ontario, Canada). The antihuman FVIII antibodies used for assay were not cross-reactive with murine FVIII. VWF activity (VWF:Ac) was determined with the INNOVANCE VWF Ac Kit (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany) on a BCS XP coagulation analyzer (Siemens Healthcare Diagnostics). FVIII activity was quantified by one-stage clotting assay with Pathromtin SL, human FVIII-deficient plasma and calcium chloride solution from Siemens Healthcare Diagnostics on a BCS XP coagulation analyzer and chromogenic substrate assay with the Coamatic factor VIII test (Chromogenix Instrumentation Laboratory SpA, Milan, Italy) using a microplate method. Both the one-stage and chromogenic assays are able to detect murine as well as human FVIII activity [18,22].

Statistical analysis

Data were analyzed using SAS version 9.3 (SAS Institute Inc., Cary, North Carolina, USA) and R version 3.0.2 (The R Foundation for Statistical Computing, Vienna, Austria) statistical software. Trough FVIII and VWF were analyzed by linear regression. Slopes of dose-to-dose change in trough levels and mean trough values after the first and last infusions with corresponding 95% confidence intervals (CIs) were calculated.

Pharmacokinetic parameters were estimated under a one-compartment model. The area under the time–concentration curve (AUC) over the period of observation was computed in accordance with the linear-up log-down trapezoidal rule and then extrapolated to infinity by means of the regression model for the terminal phase. Terminal half-life ($t_{1/2}$) was determined by log-linear regression using the points of the terminal phase selected by the adjusted R^2 criterion. Additional pharmacokinetic parameters determined were maximum concentration (C_{max}), clearance, mean residence time (MRT) and volume of distribution at steady state (Vd_{ss}). Difference in peak plasma level was assessed by *t* test for grouped data.

Results

Factor VIII trough

Over the course of the four sequential 150-IU/kg VWF:RCo infusions at intervals of 3 h (Fig. 1), trough FVIII levels in the Wilate group increased at the rate of 7.8 IU/dl per dose ($P < 0.001$). Change in trough FVIII was not significant ($P = 0.058$) for the Haemate P/Humate P group. The difference between the slopes of the two groups was not significant ($P = 0.25$).

After the first infusion, trough FVIII:Ag level in the Wilate group (31.7 IU/dl) exceeded that in the Haemate P/Humate P group (17.4 IU/dl) by 82% ($P = 0.017$). After the final Wilate infusion, trough FVIII:Ag (55.1 IU/dl) remained significantly higher ($P < 0.001$) vs. the Haemate P/Humate P group (30.2 IU/dl).

Factor VIII trough results determined by one-stage and chromogenic assays were generally similar to those for FVIII:Ag (Table 1). With both the one-stage and chromogenic assays, trough levels after the first and last infusions were higher in the Wilate group. With the exception of the comparison between troughs after the first infusion by one-stage assay, all those trough differences were statistically significant (Table 1).

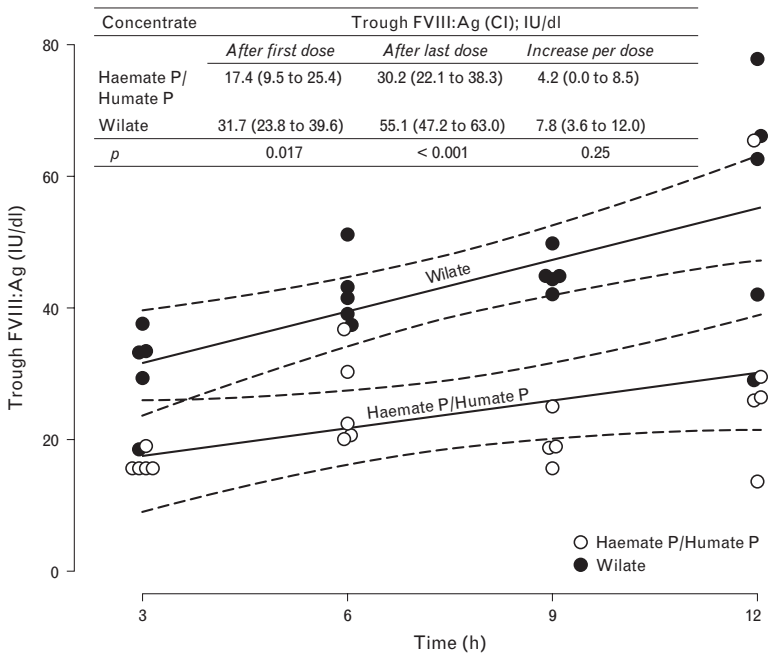
von Willebrand factor trough

Both the magnitude of and rate of change in trough VWF:Ag over the course of the sequential infusions showed minimal difference between the groups. The regression curves were essentially superimposable (Fig. 2). Nor were any differences evident in the magnitude of or rate of change in trough VWF:Ac (Table 2).

Factor VIII pharmacokinetics

After a single 250-IU/kg VWF:RCo infusion, peak FVIII:Ag was higher by 387 IU/dl (95% CI 13–762 IU/dl; $P = 0.047$) in animals receiving Wilate than those receiving Haemate P/Humate P. Higher FVIII:Ag levels persisted in the Wilate group until 6 h. AUC, which reflects cumulative exposure to FVIII, was twice as great after the single infusion of Wilate as Haemate P/Humate P (Fig. 3). FVIII:Ag clearance, MRT, $t_{1/2}$ and Vd_{ss} were

Fig. 1



Trough levels of FVIII:Ag after four repeated 150-IU/kg VWF:RCo infusions of Haemate P/Humate P or Wilate 3 h apart. Data points show individual animal values. Solid lines correspond to best fit trajectories by linear regression. Dashed lines depict CI of regressions. CI, 95% confidence interval; FVIII:Ag, factor VIII antigen; VWF:RCo, von Willebrand factor:ristocetin cofactor.

Table 1 Factor VIII trough levels determined by one-stage and chromogenic assays

		Trough FVIII (95% CI)		
Assay	Concentrate	After first dose	After last dose	Increase per dose
One-stage (% of normal)	Haemate P/Humate P	108.1 (93.5 to 122.6)	86.3 (72.1 to 100.5)	−7.2 (−14.9 to 0.4)
	Wilate	121.3 (107.8 to 134.8)	118.7 (105.3 to 132.2)	−0.8 (−8.1 to 6.4)
	P	0.20	0.003	0.24
Chromogenic (IU/dl)	Haemate P/Humate P	29.4 (19.8 to 39.0)	27.1 (17.3 to 36.9)	−0.8 (−5.9 to 4.4)
	Wilate	48.4 (38.9 to 58.0)	48.4 (38.9 to 58.0)	0.0 (−5.1 to 5.1)
	P	0.009	0.004	0.83

95% CI, 95% confidence interval; FVIII, factor VIII.

similar between the two groups (Fig. 3). Comparable pharmacokinetic results were obtained with one-stage and chromogenic assays of FVIII (Table 3).

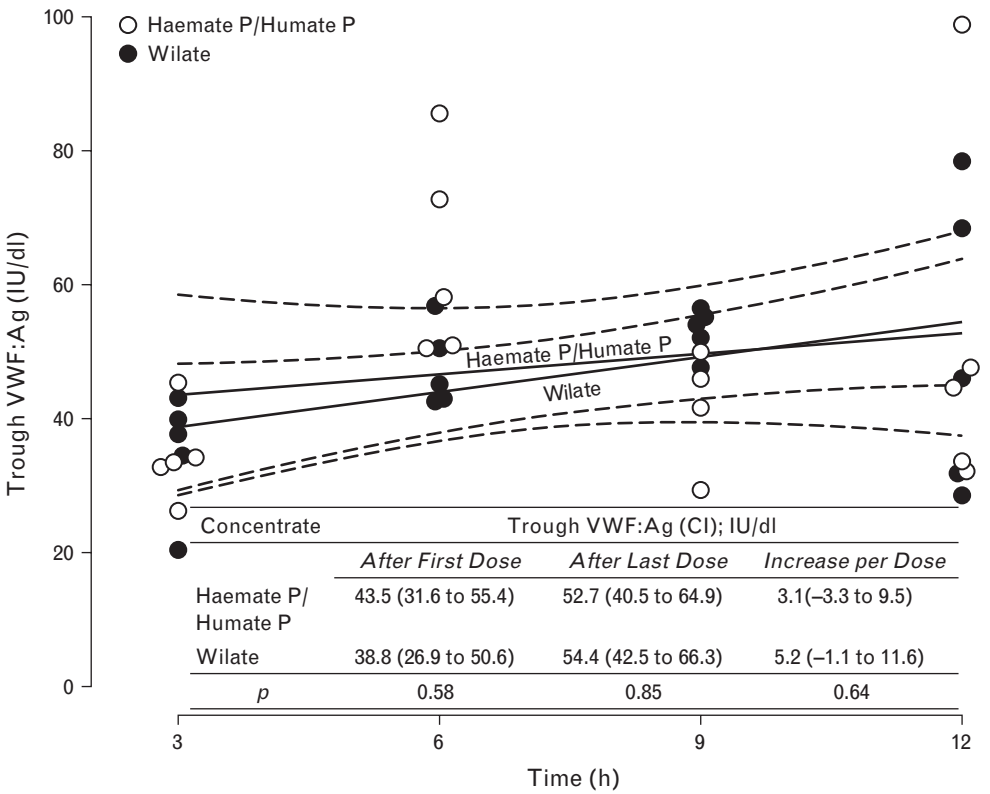
von Willebrand factor pharmacokinetics

The temporal profiles of VWF:Ag concentration after a single 250-IU/kg VWF:RCo infusion coincided tightly between the Haemate P/Humate P and Wilate groups (Fig. 4). Accordingly, pharmacokinetic parameters for VWF:Ag were similar between the groups (Fig. 4).

Discussion

In this mouse model study, two VWF/FVIII concentrates promptly and effectively corrected VWF deficiency and maintained stable target plasma VWF levels over the course of multiple infusions. In contrast to their similar effects on plasma VWF, these concentrates differed markedly in their impact on plasma FVIII. Use of concentrate with a low VWF:FVIII ratio, Wilate, resulted in 82% higher trough levels after the first dose than the high ratio concentrate, Haemate P/Humate P, and this significant difference persisted through the last dose. A statistically significant progressive increase in plasma FVIII from dose to dose, indicative of accumulation, was observed in animals receiving Wilate, but not Haemate P/Humate P. This FVIII accumulation was evident both by FVIII:Ag assay specific for human FVIII and by one-stage and chromogenic assays capable of detecting

Fig. 2



Trough levels of VWF:Ag after four repeated 150-IU/kg VWF:RCo infusions of Haemate P/Humate P or Wilate 3 h apart. Graphic conventions as in Fig. 1. CI, 95% confidence interval; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor:ristocetin cofactor.

Table 2 Trough von Willebrand factor activity levels

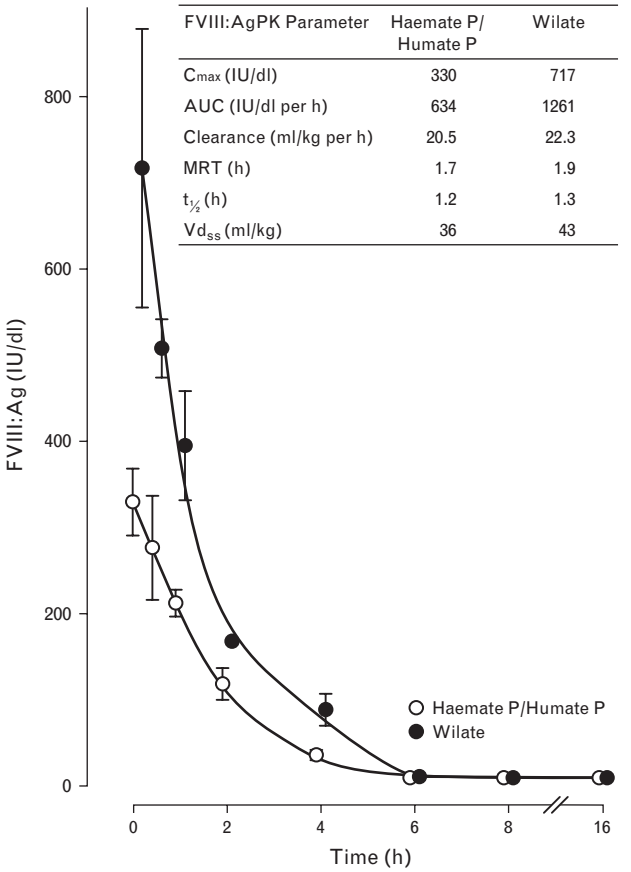
Concentrate	Trough VWF:Ac (95% CI; % of normal)		
	After first dose	After last dose	Increase per dose
Haemate P/Humate P	46.7 (33.8 to 59.7)	35.4 (22.2 to 48.7)	−3.8 (−10.7 to 3.2)
Wilate	39.2 (26.2 to 52.1)	46.1 (33.2 to 59.0)	2.3 (−4.6 to 9.2)
P	0.42	0.27	0.23

95% CI, 95% confidence interval; VWF:Ac, von Willebrand factor activity.

endogenous murine FVIII in addition to administered exogenous human FVIII.

The present data are in accord with a previous study of repeated VWF/FVIII concentrate infusions in normal rabbits [17]. Compared with Haemate P/Humate P, Wilate also produced significantly higher trough FVIII levels after the first infusion in the rabbit which persisted through the last infusion. The normal rabbit is, however, not a model for VWD *per se*, since endogenous VWF levels are not decreased. The present findings in a mouse model of type 3 VWD indicate that FVIII trough differences between Haemate P/Humate P and Wilate are independent of endogenous VWF.

Fig. 3



Time course of changes in mean FVIII:Ag levels after a single 250-IU/kg VWF:RCo infusion of Haemate P/Humate P or Wilate. Error bars represent SD. AUC, area under the time–concentration curve; C_{max}, maximum concentration; FVIII:Ag, factor VIII antigen; MRT, mean residence time; PK, pharmacokinetic; $t_{1/2}$, terminal half-life; Vd_{ss}, volume of distribution at steady state; VWF:RCo, von Willebrand factor:ristocetin cofactor.

The results of this study are perhaps not unexpected in view of the differing proportions of FVIII in the two tested concentrates. That expectation has, however, been called into question by theoretical modeling suggesting greater accumulation of Haemate P/Humate P than Wilate [24,25]. Neither this study nor the earlier normal rabbit study [17] supports the results of the theoretical modeling.

The possibility that VWF/FVIII concentrate infusion to restore and maintain adequate plasma VWF may be accompanied by elevated FVIII levels is a source of clinical concern. FVIII might potentially accumulate over the course of repeated infusions prompted, for instance, by surgery. Additionally, baseline FVIII levels in some VWD patients are only modestly reduced, and elevated FVIII might arise over a comparatively short course of treatment with VWF/FVIII concentrate.

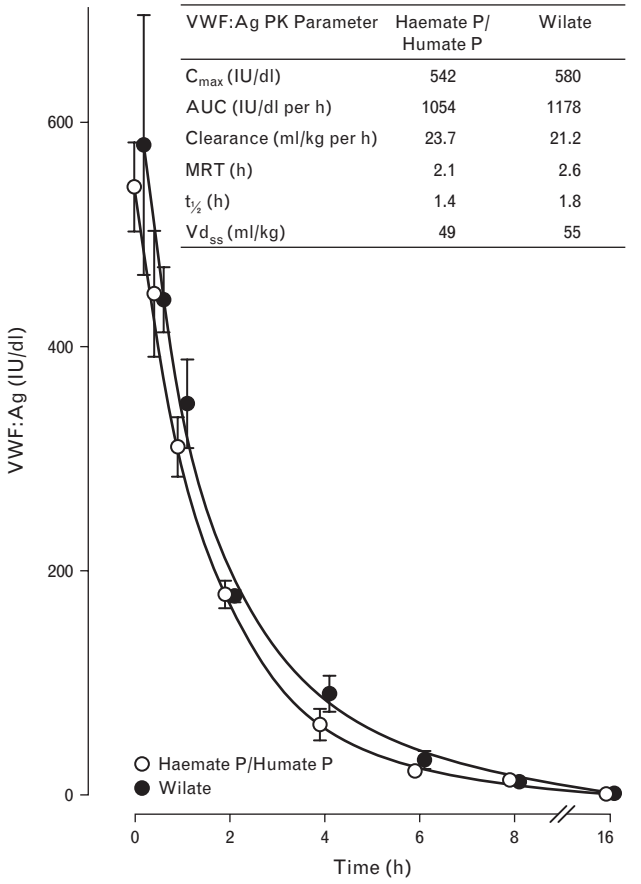
The utility of a high VWF:FVIII ratio in maintaining target VWF while avoiding elevated FVIII has been demonstrated by clinical studies of Haemate P/Humate P in surgery and long-term prophylaxis [8,26,27]. In a prospective study of 28 surgical patients receiving repeated Haemate P/Humate P infusions to maintain more than 50% VWF levels postoperatively, mean trough plasma VWF of 62–73% was maintained, while mean trough FVIII ranged between 114 and 136% [8]. FVIII levels during repeated Haemate P/Humate P doses for surgery and secondary long-term prophylaxis remained below 180 IU/dl in a retrospective cohort study [27]. In a

Table 3 Factor VIII pharmacokinetic parameters determined by one-stage and chromogenic assays

Parameter	One-stage		Chromogenic	
	Haemate P/Humate P	Wilate	Haemate P/Humate P	Wilate
C_{max} (IU/dl)	387	665	239	562
AUC (IU/dl per h)	697	1104	536	858
Clearance (ml/kg per h)	18.7	25.5	24.3	32.8
MRT (h)	2.1	1.9	2.2	1.7
$t_{1/2}$ (h)	1.4	1.3	1.5	1.2
Vd_{ss} (ml/kg)	39	48	54	55

AUC, area under the time–concentration curve; C_{max}, maximum concentration; FVIII, factor VIII; MRT, mean residence time; PK, pharmacokinetic; $t_{1/2}$, terminal half-life; Vd_{ss}, volume of distribution at steady state.

Fig. 4



Time course of changes in mean VWF:Ag levels after a single 250-IU/kg VWF:RCo infusion of Haemate P/Humate P or Wilate. Error bars indicate SD. AUC, area under the time–concentration curve; C_{max}, maximum concentration; MRT, mean residence time; PK, pharmacokinetic; $t_{1/2}$, terminal half-life; Vd_{ss}, volume of distribution at steady state; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor:ristocetin cofactor.

retrospective study of major surgery patients, mean trough FVIII:C equaled 104.2 IU/dl during daily perioperative monitoring [26].

The feasibility of maintaining normal FVIII during repeated infusions of low VWF:FVIII ratio concentrate remains to be established. In a preliminary report from two centers on patients receiving Wilate for surgery or acute bleeding, FVIII:C trough level increased by a mean of 31.9 IU/dl among those treated for at least 3 days, although the increase was not statistically significant [28]. The report did not specify how many patients received repeated infusions, the number of those infusions or the magnitude of attained FVIII:C trough levels.

The observed FVIII trough differences in VWF-deficient mice were paralleled by differences in acute pharmacokinetics, as shown in Fig. 1. After a single infusion, both C_{max} and AUC of FVIII after infusion of Wilate were twice as great as of Haemate P/Humate P. In two randomized clinical trials, greater C_{max} and AUC have been shown with either infusion of Wilate [29] or another low VWF:FVIII ratio concentrate produced by recombinant methods [30] as compared with Haemate P/Humate P.

Unfortunately, standard pharmacokinetic models are inadequate for analyzing FVIII levels in VWD patients [31]. Ongoing endogenous synthesis of FVIII results in a characteristic early plateau in FVIII levels, independent of the VWF:FVIII ratio in the administered concentrate [6,8,23,30,32,33], which is thought to reflect stabilization of endogenous FVIII by infused VWF. Consequently, FVIII levels cannot be reliably predicted from pharmacokinetics and must be monitored. The recommended frequency of monitoring is every 12 h on the day of infusion and every 24 h afterward [3,7,14,34]. Reducing the frequency of VWF/FVIII concentrate administration has been advocated, should FVIII levels exceed 150 IU/dl [27].

The need for such precautions notwithstanding, it should be born in mind that thromboembolic events in VWD patients receiving VWF/FVIII concentrates have been rare. The long-term safety record of such concentrates [35] has been further strengthened by a number of recent studies in which no thromboembolic events were encountered [30,36–38].

One limitation of the present study was the lack of data on thromboembolic safety. Furthermore, while the VWF-deficient knockout mouse has been well established as a model of severe VWD, its clinical applicability for comparing different therapeutic interventions is less certain. Because VWF is cleared more rapidly from the plasma of the VWF-deficient mouse than the patient with VWD, it was necessary to infuse the test VWF/FVIII concentrates with a shorter dose-to-dose interval than would typically be employed in the clinic. Nevertheless, the appropriateness of dosing every 3 h in this study is indicated by the half-life of 1.2–1.3 h for FVIII in VWF-deficient mice (Fig. 3) and 1.4–1.8 h for VWF (Fig. 4), and the lack of significant FVIII accumulation after repeated doses of Haemate P/Humate P (Fig. 1) or of VWF accumulation after repeated doses of either concentrate (Fig. 2). Also, as indicated in Fig. 1, a clear-cut characteristic early plateau in FVIII levels was not apparent in the knockout mouse, although human VWF has been shown capable of stabilizing murine FVIII *in vivo* [22,39].

Clinical studies directly comparing Haemate P/Humate P and Wilate after repeated doses have not been reported. Much needed controlled data are furnished by the present investigation, as well as the previous normal rabbit study [17]. This head-to-head comparison of two VWF/FVIII concentrates in an animal model of severe VWD provides evidence that a low VWF:FVIII ratio may be associated with accumulation of FVIII during repetitive infusions. Clinical studies are needed to delineate the trough FVIII levels resulting from multiple infusions of low VWF:FVIII ratio concentrates.

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Conflicts of interest

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