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C O N F I D E N T I A L

REPORT OF SNBTS VISIT TO CRTS LILLE

9 - 11 JULY 1990

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C:/WS5/REPORTS/FOSTER/KG070016.090

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SUMMARY

1. There has been considerable investment in the manufacturing facilities and infrastructure at Lille. However manufacturing practice and organisation is generally poor.

2. Some aspects of the Lille plant were impressive (eg. freeze drying facility, autoclaves) and appropriate PFC personnel (eg. Perry, Lines, Walker) should visit Lille to see these facilities.

3. Methods for the quantitation of FVIII are unusual and there is evidence that total protein measurements are incorrect. Consequently FVIII yield and purity may be overestimated by about 30% and 2-3 fold respectively.

4. Safety of the FVIII product is questionable because:-

   - the virus inactivation procedure is insecure
   - the safety/toxicity of the chromatography gel is unknown
   - at least some FVIII batches are unstable/activated

5. The factor VIII process suffers from:-

   - unreliable and insecure supply of the essential reagent
   - variable (diminishing) performance from run-to-run
   - time-consuming and expensive operation
   - lack of knowledge concerning the separation mechanism involved

6. Further work is required to bring the Lille process to acceptable standards of product safety, product quality and process performance.

   The SNBTS should collaborate with Lille in these areas.

7. Lille high purity Factor VIII has been used to treat 300 haemophiliacs in France for about 2 years. Unfortunately there appears to have been no clinical assessment or analysis of this patient group.

   The SNBTS should encourage CRTS Lille to study this database to determine whether or not any clinical benefit can be associated with this product.

1. INTRODUCTION

The objective of the visit was primarily to learn about and observe the Lille process for FVIII manufacture. During the visit discussions were held with:-

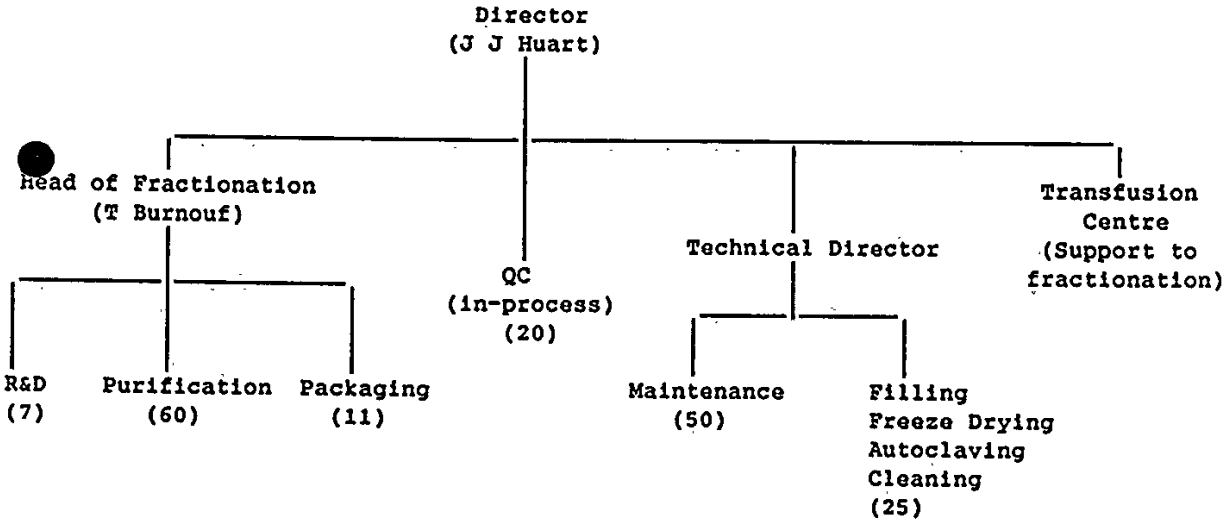
Dr T Burnouf	(Head Fractionation)
Mme A Faucompre	(Process Manager - FVIII, VWF, Fibrinogen, Fibrin Glue)
M Leprince	(Assistant Process Manager)
Dr C Mazurier	(QC - final product)
Dr C Michalski	(QC - in process) (Dr Pepper and Prowse only)
Dr M Burnouf - Radosevich	(plasma product R&D)

This report presents the information with which we were provided (sometimes conflicting) and our own observations and interpretation. During the visit we also had the opportunity to briefly view the wider manufacturing facility and information on this is also included.

2. RESOURCES AND FACILITIES (GENERAL)

2.1 Staffing

The Fractionation Centre is closely associated with the Transfusion Centre both of which come under the management of Dr J J Huart (total of about 700 staff). Some transfusion departments also provide a service to the Fractionation operation (eg. final product assays) and the general organisational structure appeared to be:-



Conditions of employment are established locally under a CRTS Board (includes local dignitaries) and a two shift system is operated (8am - 4pm/2pm - 10pm) with a caretaker shift through the night.

2.2 Facilities

The Fractionation Centre was spread across 2 adjoining buildings and over a number of floors. A third building is under construction.

The total floor area available for production was said to be 4000m^2 but the layout of the operations did not follow any obvious pattern (none of us could sketch a floor plan even though we spent two days in the Centre). As different product operations were spread all over the site there was considerable movement of materials (supernatants) by pushing tanks along corridors and up and down lifts.

The most notable features of the Centre were:-

(A) Its large size (eg. the packaging area was bigger than PFC's main fractionation hall)

(B) The large quantity of equipment
- countless stainless steel tanks
- ultrafiltration systems for virtually every product line
- numerous chromatographic columns (up to 100l)

(C) Four very large autoclaves capable of taking tanks.

(D) An impressive freeze drying suite with a large number of double-ended Usifroid driers interfaced with sterile dispensing rooms.

An adjacent control room contained:-
- normal control modules and chart recorders
- PC/VDU's for monitoring drying cycles
- PCU/VDU for alarms and fault diagnosis

The cost of this drying suite was estimated at £0.2M/drier two years ago. (It was not clear exactly how many production freeze driers there were; minimum 6, maximum 11)

(E) 3 sterile dispensing rooms
- 1 for albumin and crystalloids and 2 used for freeze dried products (all products with in-process VI treatment)

(F) A large packaging area which was largely automated.

(G) Pallet jacks with loadcells were in widespread use for weighing the contents of vessels up to 200l in size.

2.3 GMP (general)

2.3.1 Good Points

(A) neat clothing stations in change areas (except for entry to VI suite)

(B) "Wheelie" bins (large and small) used for rubbish collection - very clean and tidy

(C) Strong, high-quality overshoes

2.3.2 Bad Points

(A) Poor building design and layout

(B) Tiled floors with dirty grouting, no stick mats, no step-over barriers at key points (eg. VI suite)

(c) Considerable transport of materials and people (eg. use of lifts)

(D) Poor clothing control (eg. everyone walks over pile carpets in clean overshoes). Production clothing worn in coffee area, etc.

(E) Ancillary equipment in very dirty state (eg. wheels of pump trolleys)

(F) Multiple products being processed simultaneously in same confined area (eg. FVIII, VWF, Fibrinogen, Fibrin Glue)

(G) Multiple batches being inspected and packaged simultaneously

(H) Multiple batches in packaging and inspection left half-finished and insecure over-night (including labels and packaging)

(I) Reject materials (eg. IV IgG rejected because of hypotensive reactions) left in Production area unlabelled and insecure (to be used for development experiments in Production)

(J) Process reagents taken directly from manufacturers container (eg. $Al(OH)_3$, TNBP/Tween)

(K) Inadequate documentation eg. PSBR for FVIII process (from plasma to sterile filtered product) Lille 10 pages; PFC 30 pages

(L) Virus inactivation suite insecure (see below)

(M) Open water baths used for incubation in processing areas.

2.3.3 Comment

The Lille centre has still to adopt the standards of GMP expected from the plasma fractionation industry in the UK and USA. This covers all aspects of manufacturing, including the quality of the facilities, the organisation and control of processes and the control of personnel.

2.4 Health and Safety

The attitude to health and safety appeared slack eg:-

(A) Safety spectacles were not being worn even for obviously hazardous tasks (eg. taking plasma 'core' samples by machine tool)

(B) Production and laboratory clothing was worn in the coffee area

(C) Only surgeons gloves were being worn for the cutting of cryoprecipitate slabs using very sharp long bladed "kitchen" knives

2.5 Some Comparisons With PFC

		LILLE	PFC
(A)	Plasma throughput kg/yr	150 000 +	80 000
(B)	Production floor area, m^2	4 000	1 000
(C)	Shift working	YES	NO
(D)	Number of staff		
	- Purification	60	23
	- Filling, drying, autoclaving, etc.	25	32
	- Packaging	11	12
	- QC - in-process	25	
	- final product	?	
	- total QC	?	32
	- Maintenance	50	30 (incl.P.C)
	- R&D (plasma products)	7	13
(E)	Number of V.I suites	3 (+1)	0
(F)	Number of dispensing suites	3	1
(G)	Number of product freeze driers		
	- Production	6 (+5?)	3
	- R&D	2	6

3. FACTOR VIII PROCESS

METHODS	COMMENTS
3.1 Assays	
(A) VIII C	
(i) Potency derived from mean of 1-stage and chromogenic assays using plasma standard. (ii) All dilutions in haemophilic plasma. (iii) Value of plasma standard assigned by Lille/Paris on basis of 1-stage assays (664900.1 : 1.09 u/ml) (iv) Same methods used for in-process and final product samples. Done by different labs with little inter-lab comparison. (v) Comparisons done on final product between Lille and Paris. Paris assays Paris high and Lille assays Lille high r = 0.84 (1-stage) r = 0.68 (chromogenic) (vi) Sometimes discrepancies between 1-stage and chromogenic. Believed to be due to activation (Mazurier)	(a) The methods used at Lille are unusal and differ from PFC in eg:- - the type of standard used - the procedure for assigning a value to the standard - the nature of the diluent (b) These methods were justified on the grounds that they match the clinical experience. The data supporting this (see appendix 8.5) is very flimsy and important controls are missing. (c) Whatever the merits of the Lille approach it is clear that their methods will give higher VIIIC results than those used by PFC (and control authorities), possibly by as much as 30% (even in the absence of activation). (d) Yield comparisons will have to take this into account.
(B) Total Protein	
(i) Total protein is measured by the Bradford assay using BSA as a standard.	(a) The data that we were shown provides further evidence that the method used by Lille to determine total protein may be incorrect and may underestimate the protein content substantially.

METHODS	COMMENTS
(ii) Comparisons with other protein assays are now being undertaken. The general trend is:- Lowry ≡ Bradford < BCA eg. Protein (mg/ml) Sample Bradford BCA 1 0.18 0.42 2 0.14 0.37 3 0.73 1.25 4 0.25 0.45 5 0.39 0.59 6 0.21 0.38 Mean 0.317 0.577 Ratio 1 1.82	(b) Taking protein and VIIIC determinations together it is possible that specific activities previously quoted by Lille may be 2-3 fold too high.
3.2 <u>Plasma</u> (i) FFP is both recovered and 'pheresis plasma (latter costs FF 300 per donation) (ii) Stored in -35°C room unboxed. Single donations stored either in mesh baskets or hung on racks. (iii) Frozen "core" sample taken from 20 donations per plasma pool for VIII C assay. (iv) VIII C values given as:- 0.8 - 1.2u/ml (Mazurier-data) 0.55 - 0.80u/ml (Burnouf-comment)	(a) The plasma cold storage area seemed spaceous and well organised and was frost free.

METHODS	COMMENTS
3.3 Thawing & Cryoppt Collection	
(i) Plasma removed from -35°C room to 0°C room at 5pm for 7am start the next day. Final plasma temperature believed to be about -6°C.	(a) The procedures used here were fairly traditional with less control and a lower yield than we would expect at PFC.
(ii) Thawing area (+10°C room) contains bandsaw for pack removal, thawing vessel (500l) conditioning tank and 3 Sharples centrifuges (restricted to 4.5kg each)	
(iii) 600-800l pools of plasma processed 3-4 days/wk. Thawing is semi-batch; conditioning (+1°C) is used to redissolve some unwanted protein; centrifuge feed is 500l/hr per centrifuge.	
(iv) In-line strainers used to catch plastic etc.	
(v) Cryoppt aliquoted into bags, heat sealed, rolled into thin slab and stored (-35°C) for >1 month.	
(vi) Cryoppt is 8.5 g/l plasma FVIII Yield of extracted cryo said to be:- 400 - 600 u/l (Burnouf-comment) 392 iu/l (Faucompre-data, n=1)	
(vii) Cryosupernatant VIIIC 0.16-0.32u/ml (Mazurier) Bacteria 45-60 cfu/ml (Burnouf)	

METHODS		COMMENTS	
3.4	Cryoprecipitate Processing (see SOP, appendix 8.2)		
(i)	Frozen cryoppt held at 20°C for 3 hrs, sliced using sharp knives; tipped into H_2O (28.7°C, 4 l/kg cryo) with gentle stirring. (Note: Octapharm said to include 1% Ethanol). Lille solution includes heparin (3 u/ml).	(a)	Local application of traditional methods. No new information.
		(b)	Extraction vessel, coolant control and quantitation (pallet jacks with load cell) all good features.
(ii)	Temperature brought to 25°C by feeding tank jacket with controlled water supply (from +40°C/+4°C supplies) using feedback control loop measuring process temperature.	(c)	Manual procedures and general control not so good. Heavy and expensive reliance on filters.
		(d)	No apparent yield or quality advantage over PFC methods.
(iii)	pH dropped to 7 (0.1M acetic acid), $Al(OH)_3$ tipped in (not sterilised) pH dropped to 6.5 (0.1M acetic acid tipped in), temperature reduced to 15°C (over 20 minutes). Final temperature 13-14°C.	(e)	The length of time taken to thaw the cryoppt is suspect and may contribute to problems of activation during subsequent processing.
(iv)	Solid phase allowed to sediment. Sludge collected in 3 litres bags and centrifuged in swing-out buckets.		
(v)	Supernatants pooled and filtered very slowly through:- cloth; 6μ (30"), 2μ (12") 1μ (12") and 0.46μ (12")		
(vi)	Stage yield given as:- 85% (Burnouf-comment) 75% (Faucompre-actual, n=1)		

METHODS	COMMENTS
3.5 <u>Virus Inactivation</u> (see SOP, appendix 8.2) (i) Extraction vessel cleaned and rinsed while centrifugation and filtration (above) in progress. Returned to be used for 1st stage of VI treatment. (ii) $CaCl_2$ (100mM) added to solution (1mM) to provide stability over VI period (Lille advised of this by NYBC !!!) pH brought to 7. (iii) TNBP/Tween taken from manufacturers bottles, mixed together at 45-50°C. Added to FVIII solution and held for 1hr before transferring to 2nd tank held in VI suite (6hrs) (iv) Stage yield: Mean of 5% increase in VIIC iu/ml (? effect of reagents on assay). Line losses not given.	(a) Some procedures carried out poorly (eg. $CaCl_2$ and 0.5M NaOH poured directly from measuring cylinders) (b) Not clear how key reagents (TNBP, Tween) are quality controlled before use. (c) Disposal of TNBP and Tween is down the drain??
3.6 <u>V.I "Containment" Facility</u> (see sketch, appendix 8.1) (i) Used for 2nd stage of FVIII VI (6hrs in stirred tank at RT) and subsequent chromatographic purification (below). (ii) Consisted of a partitioned area beyond the cryoppt processing area. (iii) Entrance was through a changing area. All equipment and supplies passed through the same entrance. (iv) Emphasis was on staff clothing discipline (but even this did not always work) with no attempt to operate a truly closed system.	(a) Arrangements for segregating and containing VI treated materials was insecure with risks of viral contamination via:- - equipment - components - tools - untreated materials - personnel (b) GMP was generally poor, eg. - open water baths - congestion - multiple processes running simultaneously - many manual procedures with "dirty" (gloved) hands.

METHODS	COMMENTS
(v) All equipment and components had passed through plasma and cryo processing areas to get here, with the possibility that their exterior surfaces could be contaminated (especially tank wheels)	
(vi) Staff were tempted to share miscellaneous items (eg. scissors) with those working in adjacent areas.	
(vii) Containers of TNBP and Tween were stored inside the VI suite suggesting that untreated materials are brought here for treatment.	
(viii) The processing of freeze dried Fraction I for fibrin glue was one possible example of this. Powder was emptied from trays within the VI suite, leaving particles spread over working surfaces. Subsequent possibility of transfer to FVIII eluate and to downstream fibrin glue was observed.	

METHODS		COMMENTS	
3.7	Chromatography (see SOP, appendix 8.2)		
(i)	The chromatography step uses DEAE-Fractogel packed into 2 separate 20 litre columns (11 litre beds) which are run in parallel from a common process supply. Scale-up to larger columns had been attempted but appeared not to have been successful.	(a)	The overall process was lengthy (about 8 hours from loading to elution) and, with largely manual operation, afforded opportunity for error (eg. forgetting to collect the VWF product) and for the introduction of bacterial and possibly viral contaminants.
(ii)	Operation of the columns was manual and the columns are repacked every 2-3 runs to achieve improved cleaning.	(b)	Operation of the columns in parallel meant that different flow regimes (hence separation) were being achieved in each column (as the pressure drop over each column was different) This is poor process design.
(iii)	The gel is manufactured by TSK (Japan) but is supplied by Merk (Germany).		
(iv)	Merk appear to be attempting to manufacture the gel themselves (? under sub-licence) but the batches they have prepared (modified) have been unsuitable for the Lille process (low yield)	(c)	The mean stage yield appears to be relatively low and run-to-run variability must be a manufacturing nightmare.
(v)	The separation mechanism is not understood and relevant gel characteristics cannot be specified. 3/10 TSK (Japan) batches supplied by Merk have not been suitable (low stage yield). Comparative performance tests are carried out by QC on every batch prior to selection.	(d)	Supply of the chromatography gel is unreliable and insecure and is a cause of major concern; "there is a real danger that they (Merk) will not be able to supply the gel" (Burnouf).
(vi)	The performance of the gel decays progressively from 85% to 60% stage yield over 15 runs, when it is discarded. Hence the mean stage yield is 72% (the "missing" FVIII cannot be accounted for and the mechanism of loss is not known). This falls by a further 10% if the S/D step is omitted.	(e)	The apparent indifference to the need to obtain data on gel chemistry/stability/safety/ toxicity was astonishing, particularly when there is no solute removal step (eg. UF) following chromatography.
(vii)	Information on the toxicity and safety of the gel has not been sought.		

METHODS	COMMENTS
3.8 Sterile Filtration (see SOP, appendix 8.2)	
(i) The product eluate is sterile filtered (0.2μ) in the VI suite prior to transfer to the dispensing suite some distance away (different floor in ? different building).	(a) The FVIII solution appears to be terminally filtered within the suite.
	(b) This solution must then be transported to the dispensing area and presumably then transferred in a sterile manner to another closed vessel within the sterile dispensing area.
(ii) The sterile filter (Pall 0.2μ) is primed with 400ml of 4.5% Human Albumin to prevent loss of Factor VIII by adsorption. There is some evidence that this may not be required if the Durapore (Millipore) filter is used instead.	
	(c) The opportunity for residual volume losses would seem to be high with this procedure.
(iii) The stage yield was not available.	
3.9 Dispensing	
(i) FVIII solution is dispensed either as 10ml or 20ml in 50ml vials.	(a) We were not able to observe any dispensing being carried out or to see clearly into the room.
(ii) This is done in either of 2 sterile rooms which interface with freeze drying plant.	(b) The segregation of terminal VI treated products (eg. albumin) from in-process treated products (eg. FVIII) seemed to be being carried out possibly more by chance than by design (although it was not clear in which room I.M IgG was dispensed).
(iii) Other products are also dispensed in these rooms (eg. FIX, IV IgG, α_1-AT, etc.) but the risk of any cross-contamination was said to be avoided by the use of disposable tubing.	

METHODS	COMMENTS
3.10 Freeze Drying (see appendix 8.3)	
(i) The freeze drying cycle appeared to include the following notable features:- - supercooling - tempering of frozen product - short total freezing time - low product temperature held in primary drying (-40°C to -50°C) despite high shelf temperature - fairly aggressive primary drying - product stoppered under full vacuum	(a) The drying cycles appear to have been designed by Usifroid who have played a major role in designing and installing the plant and in training staff. (b) The ability to maintain a very cold product temperature during primary drying with a relatively warm shelf seemed initially to be a contradiction but this may be explained by the unusual design of the freeze driers. The condenser coils are situated at either side of the shelves within the drying chamber. Hence the condenser probably plays a major role in cooling the drying chamber (by radiant heat transfer).
(ii) Stoppers used were from Pharmagummi (1292 chlorobutyl PH2150). The bungs are received siliconised, are washed under filtered air, double wrapped and autoclaved at 121°C for 40 mins.	
(iii) All driers are calibrated for temperature and pressure every 6 weeks. This involves a 3-day shut down of the sterile dispensing room.	(c) It would seem that instead of designing an appropriate product formulation and drying cycle suitable for standard industrial freeze driers, Usifroid have designed a special freeze drier while retaining a relatively traditional FVIII drying cycle.
(iv) Leak testing is carried out on all driers every 7 weeks using helium gas with mass spectrometry.	(d) This approach has the disadvantage that the temperature profile across the drying chamber may vary markedly (in 3-dimensions) and will be difficult to control. Evidence for this can be seen in the product temperature profile (appendix 8.3) where the temperature of the centre vial rises quickly during primary drying relative to the other vials.

METHODS		COMMENTS	
3.11	Other Product Features		
(i)	Stability		
	The product stability is assessed by monitoring VIII C (1-stage and chromogenic) at 4, 8 and 12 hours following reconstitution. Most batches said to be stable but some batches had been unstable according to:-	(a)	There are clearly problems with activation, although the extent is uncertain as instances of lower level activation may be being masked by the use of haemophilic plasma as an assay diluent.
	- 1-stage vs chromogenic discrepancy - increasing 1 stage values about 1 week after drying followed by progressive loss of activity in dried state	(b)	The unstable behaviour described to us was similar to that previously seen by ourselves and may well be a feature common to all current high purity products (Barrowcliffe et al, Lancet ii : 124, 1990). It may also explain the anomalous results observed in the BPL study.
(ii)	Osmolarity		
	The Lille FVIII has an osmolality of 550mOsm and the corresponding VWF product is 800mOsm.	(a)	The osmolality used by Lille is somewhat higher than that previously recommended to us for the long term treatment of severe haemophilia.
(iii)	Moisture		
	The moisture content of the product was said to be in the range 0.3 to 0.8%.		
(iv)	TNBP/Tween (see appendix 8.4)		
	Residual quantities of these reagents are below the "FDA limits".	(a)	It is not clear how the "FDA limits" were established and if any follow-up safety studies with haemophiliacs are underway.

4. FURTHER COMMENTS ON THE LILLE FACTOR VIII PROCESS

(i) In designing a manufacturing process for pharmaceutical infusion products it is necessary to ensure that:-

- all reagents are safe or that any potential harmful contaminants have been removed from the product
- appropriate specifications are available for all reagents (particularly those that determine a key separation process)
- the supply of essential reagents is secure

It is also desirable that the process performs in a reproducible manner and is capable of being scaled-up as required.

Currently the Lille FVIII chromatography process meets none of these conditions.

(ii) It should also be noted that the virus inactivation procedure adopted by Lille is suspect because of inadequate security downstream of the solvent/detergent treatment.

Our observation of this procedure in operation has confirmed our view that higher levels of security require the installation of a completely closed system rather than simply an additional room adjacent to the upstream processing areas. This will involve provision of a completely separate facility for all process steps downstream of the VI treatment; including autoclaves, cleaning and dispensing equipment as well as dedicated process equipment, supplies and staffing.

(iii) The FVIII product has been in clinical use for almost 2 years and some 300 patients come under the management of Lille CRTS. Data appears to be being collected on these patients but this has not been reviewed or analysed and there seems to be little or no interest in doing this (Burnouf).

5. OTHER PRODUCTS

5.1 Albumin

(i) Albumin products are prepared by cold ethanol fractionation. Fraction I+II+III and FIV are removed by depth filtration. Supernatant IV is diluted 1:1 with water and then ultrafiltered to remove ethanol and to formulate. Albumin solutions are heat-treated at 60°C in-process (in an autoclave) to enable any unstable material to be removed prior to dispensing (slowly carried out to avoid foam) and terminal heat treatment (also carried out in an autoclave).

(ii) The use of chromatography for the preparation of albumin (Martinache et al, Proc 18th Congress ISBT, p156, 1984) has been abandoned because of problems of yield, product instability and pyrogenic contamination (Burnouf). Attempts are now being made to revamp the plant for the preparation of α1-antitrypsin.

5.2 Immunoglobulin

(i) IV IgG

Lille currently manufacture 140kg/year using pH4 pepsin treatment of FII. Dose sizes are 0.5g, 2.5g and 5g with the 5g dose dried as a 100ml plug in a 150ml bottle.

A chromatographic process is currently being developed using supernatant I. The solution is expected to be S/D treated, albumin is then bound to the ion exchange column while IgG flows through. The albumin will be subsequently eluted, concentrated by UF and added to the cold-ethanol prepared fraction.

(ii) IM IgG

Ethanol is normally removed by UF except for small pools of specific IgG (eg. anti-D) where freeze drying is still used as the UF equipment is oversize.

The UF prepared products were claimed to be clinically safe with regard to fears of possible hepatitis contamination (Burnouf - note: Martinache previously stated this, but failed to provide us with any clinical data to substantiate the point).

5.3 Fibrinogen

A fibrinogen concentrate is prepared by heparin-sepharose chromatography (about 25l bed) of the flow-through of the FVIII-Fractogel step. The product is a 2g dose dispensed as a 100ml plug in a 150ml bottle.

5.4 Fibrin Glue

The Fibrin glue process involves S/D treatment of freeze dried fraction I with the S/D reagents being removed by liquid-liquid extraction using castor oil. The product is being used for the treatment of burns.

The possibility of adding growth factors is only at an experimental stage.

6. CONCLUSIONS

6.1 The facilities and equipment at Lille are extensive and are generally of a high quality.

6.2 The layout and organisation of processes is poor and many aspects of GMP are of a low standard.

6.3 The current FVIII process is flawed in a number of respects:-

- potential toxicity of chromatography gel
- variable gel performance
- concern over gel availability
- inadequate virus inactivation process
- insufficient follow-up of patients

6.4 The FVIII process is relatively expensive in terms of

- process time required
- the cost of process reagents
- QC input

6.5 The FVIII process has no particular yield advantages (this area has been confused by the use of local assay methodology instead of internationally agreed procedures).

6.6 The FVIII instability/inactivation is a problem but the extent is not clear.

6.7 The FVIII purity is uncertain but is probably substantially less than previously claimed.

7. RECOMMENDATIONS

7.1 Further visits to Lille should be arranged for PFC staff to view the freeze drying plant, the autoclaves and other interesting aspects of the plant.

7.2 The SNBTS should collaborate with CRTS Lille on ion exchange purification of FVIII to:-

(i) characterise and specify Fractogel
(ii) determine the basis of the separation
(iii) define the potential for activation
(iv) characterise the performance of other gels of interest to SNBTS/Lille
(v) obtain clinical data on the use of a high purity product

7.3 A thorough review of our strategy for the development of a high purity FVIII product should now be undertaken.

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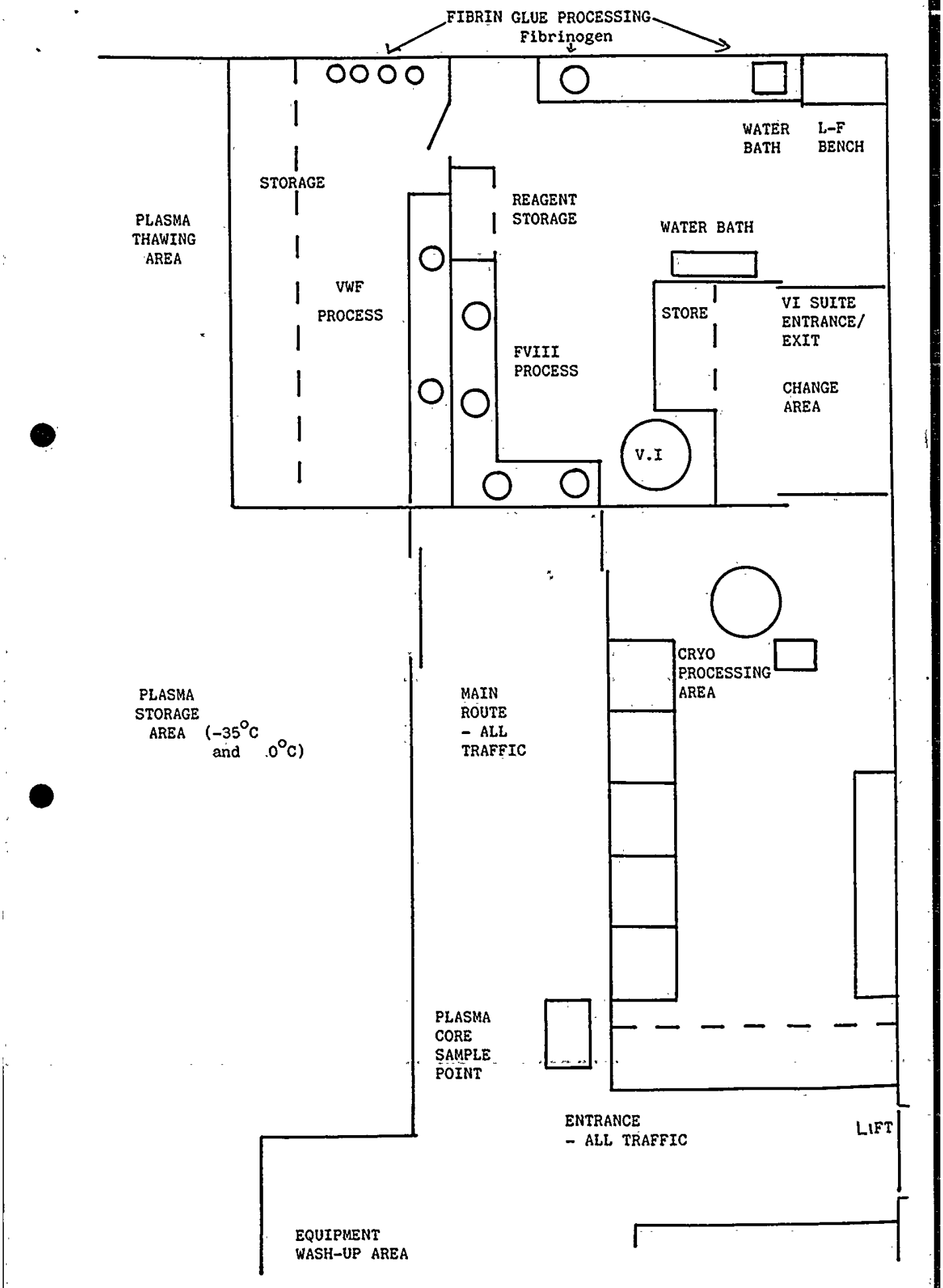
APPENDIX 8.1

Virus Inactivation "Containment" Suite

SKETCH OF VI "CONTAINMENT" AREA



APPENDIX 8.2

Method sheet (batch record) for FVIII process from cryoppt processing to sterile filtration

FRAC 03 / 02

PRODUCTION SUPER VIII THP

DATE : 10/07/90

LOT : .....

-Volume de plasma = ... litres

-Poids de cryoprécipité = ~28 kg

Numéro lot décongélation	Poids de cryoprécipité	Volume de plasma
TOTAL :	..... Kg.	..... litres

I_Remise en solution de la pâte:

-Préparer un volume d'eau PPI à raison de 4 litres par kilogramme de pâte de cryoprécipité à une température de 25 - 28° C ..... litres eau ..... °C de l'eau

-Ajouter l'héparine à raison de 5 UI/ml ..... ml

-Addition de 1% ethanol: oui - non ..... litres ETOH

- Mise en service DE LA CUVE DOUBLE ENVELOPPE N° 1:

-> Mise en service de l'armoire électrique N° 1

- Mise en place la sonde thermique dans la solution

-> affichage de la température sur le boîtier gauche à l'intérieur de l'armoire électrique (+ 26° C)

-> Ouverture des vannes circuit chaud

-> Mise en service de la pompe échangeur chaud

-> Mise en service du circuit chaud (position I : mois impairs, position II : mois pairs)

FRAC 03 / 02

PRODUCTION SUPER VIII THP

DATE : .....

LOT : .....

-> Afficher la température + 15° C sur le boîtier intérieur droit de l'armoire. Ouvrir les vannes circuit froid ..... °C initiale ..... °C finale

-> Mise en service de la pompe circuit froid (position I : mois impairs, position II : mois pairs)

- Quand la température + 15° C est atteinte (pour la solution dans la cuve) couper le circuit froid et laisser sédimenter la solution pendant 3/4 heure (Couper l'alimentation de l'armoire les différentes vannes et déconnecter la cuve double enveloppe) .....H.....mn heure d'atteinte en température

- Pomper le surnageant directement en container en filtrant par un passage sur sote monodur

- Centrifuger le précipité restant en fond de cuve JOUAN K 110 . 2700 t/mn-15°C-15mn .....H..... mn début .....H..... mn fin

- Poids de précipité ..... g

- Poids de précipité/litre de plasma: ..... g/l

- Filtrer l'ensemble du surnageant .

PALL AB3 U6 40 Z 7P \
PALL AB1 U2 20 Z 7P | (les 3 filtres montés en série)
PALL AB1 U0 10 Z 7P /

.....H..... mn début filtration

.....H..... mn fin

PALL AB2 NFE 7P

- **Recueillir le filtrat dans la CUYE DOUBLE ENVELOPPE N° 2**
  - Peser le filtrat ..... litres

- **A l'issue de la filtration, connecter la cuve n° 2 sur le système de régulation thermique**

-> Mise en service de **l'armoire N° 1**
-> Mise en place de la sonde thermique
-> Affichage de la température + 24,5° C sur le boîtier gauche de l'armoire
-> Ouverture des vannes circuit chaud
-> Mise en service de la pompe échangeur chaud
-> Mise en service du circuit chaud (I : mois impairs, II : mois pairs)

- Mettre la solution en agitation
- Température de la solution ..... °C
- **Prise de l'échantillon 3 852 :** .....

FRAC 03 / 02

PRODUCTION DE SUPER VIII THP

DATE : .....

LOT : .....

III_Inactivation virale

-Ajouter à la solution de facteur VIII ,une solution à 100mM de $CaCl_2$ jusqu'à une concentration finale de 1 mM: .....litres de filtrat x 10 = ... ml $CaCl_2$

-Ajuster le pH à 7,0-7,2 avec NaOH 0,5 M. ..... pH initial
..... ml NaOH
..... pH final

-Préparation de la solution de TNBP_Tween 80:

..... kg de filtrat x 10,1 = ... g de Tween

..... g de Tween x 4 = ... ml d'eau ppi

..... litres de filtrat x 3,03 = ... ml de TNBP

- VOLUME DE SOLUTION INACTIVATION ..... litres

PRODUITS UTILISES	REFERENCE DU PRODUIT	PROPORTION DE PRODUIT	QUANTITE TOTALITE	EMARGEMENT	EMARGEMENT CONTROLE

VISAS DES AGENTS DE PRODUCTION

FRAC 03 / 02

PRODUCTION SUPER VIII THP

DATE : .....

LOT : .....

..... °C de la solution avant addition de la pâte

- Couper la pâte en petits morceaux et la remettre en solution dans l'eau héparinée.

.....H.....mn heure addition de la pâte

- Une fois toute la pâte dans la solution :

.....°C de la solution après addition de la pâte

- Basculer la température à + 22° C sur le boîtier intérieur de façon à maintenir le produit à 20 - 25° C, sous agitation

- Commencer immédiatement l'adjonction de l'acide acétique 0,1 M.

- Ajuster le pH à 7 (6,95 < pH < 7,05) sur un laps de temps n'excédant pas 15 à 20 mn.

..... pH initial
..... litres d'acide
..... pH final

L'addition de l'acide s'effectue sous la surface du produit à un débit de 100ml/mn sous agitation continue.

.....H.....mn début addition d'acide
.....H.....mn fin
..... °C de la solution

- Prise de l'échantillon 3 85 1 .....

- Laisser la solution sous agitation pendant 15 mn avant d'ajouter le gel d'alumine

II Purification

- Ajouter 108 g de gel d'alumine par kilogramme de cryoprécipité.

..... g gel d'alumine

- Laisser 5 mn en agitation

- Ajuster le pH à 6,5-6,6 (6,50 < pH < 6,60) avec de l'acide acétique 0,1 M sur un laps de temps de 20 à 30 mn

..... pH initial
..... litres d'acide
..... pH final

.....H..... mn début addition acide
.....H..... mn fin

- Refroidir la solution à + 15 ° C dans la cuve double enveloppe

-> Au niveau de l'armoire mettre la pompe circuit chaud et l'échangeur sur la position 0
-> fermer les vannes du circuit chaud

FRAC 03 / 02

PRODUCTION SUPER VIII THP

DATE : .....

LOT : .....

- Dissoudre le tween 80 dans la quantité d'eau réchauffée préalablement à 40-50°C.
  Après dissolution, ajouter le TNBP sous agitation.

- Ajouter lentement sous agitation rapide la solution de TNBP_Tween à la solution de facteur VIII. L'addition doit durer au moins 15 mn.

  - addition TNBP-Tween .....H.....mn début
  .....H.....mn fin

- Enregistrer la température du produit: .....°C.

- Maintenir la solution sous agitation douce dans la cuve double enveloppe à 25° C (+ 24° C ≤ t° ≤ + 26° C), pendant 6 heures minimum après la fin de l'addition du TNBP_Tween.

(1 heure après l'addition de TNBP_Tween, transvaser la solution de facteur VIII dans une autre cuve double enveloppe préalablement stérilisée, placée dans la pièce d'inactivation virale).

- Le transfert du produit s'effectue à l'aide d'une pompe péristaltique équipée d'un tuyau stérilisé (ce matériel ainsi que la cuve n° 2 demeurent dans la première pièce de travail)

RAPPEL: APRES INACTIVATION, TOUT LE MATERIEL UTILISE DOIT ETRE STERILISE.

- Mise en service du circuit chaud au niveau de l'armoire N° 2 (pièce d'inactivation virale)

  -> Connection de la cuve sur le circuit (cuve stérilisée)

  -> Ouverture des vannes (cuve et circuit chaud)

  -> Affichage de la température sur le boîtier gauche de l'armoire (+ 24,5° C)

  -> Mise en service de la pompe échangeur

  -> Mise en service du circuit chaud (I : mois pairs, II : mois impairs)

  -> Mise en place de la sonde thermique

FRAC 03 / 02

PRODUCTION SUPER VIII THP

DATE : .....

LOT : .....

_IV_RELEVE DES TEMPERATURES_

HEURE	TEMPERATURE DU PRODUIT
.....H.....mn	.....°C
.....H.....mn	.....°C
.....H.....mn	.....°C
.....H.....mn	.....°C
.....H.....mn	.....°C
.....H.....mn	.....°C
.....H.....mn	.....°C
.....H.....mn	.....°C

_V-CHROMATOGRAPHIE

_1_Préparation des tampons:

	Cuve 1	Cuve 2	
-a-Tampon équilibrage:			
_N° lot tampon	:		
_Volume préparé	:		litres
_pH	:		
_Osmolarité	:		mosm/l
_Prise des échantillons 3 85 6 et 3 85 7	:		
_b_Tampon de prélavage:			
_N° lot tampon	:		
_Volume preparé	:		litres
_pH	:		
_Osmolarité	:		mosm/l
_Prise de l'échantillon 3 85 8	:		
-c- Tampon d'élution			
_N° lot tampon	:		
_Volume préparé	:		litres
_pH	:		
_Osmolarité	:		mosm/l
_Prise de l'échantillon 3 85 9	:		

FRAC 03/02

PRODUCTION SUPER VIII THP

DATE : .....

LOT : .....

2- Déroulement de la chromatographie

-a- l'injection du VIII

- Volume de facteur VIII	:	litres
- pH initial FVIII	:	
- Osmolarité FVIII	:	mosm/l
- **Prise de l'échantillon 3 85 3**	:	

- Calcul du volume de sels à ajouter :

Soit V le volume de solution de F VIII (connu)
X le volume de solution d'équilibrage concentré 10 fois (inconnu)
osm l'osmolarité de cette solution (connue)

Il faut résoudre l'équation
$(V + X)\ 387 = osm \times X$

Soit $X = \frac{387\ V}{osm - 387}$

soit dans le cas présent

$X = \frac{387\ V}{osm - 387} =$ _____ litres

- Volume de sels à ajouter	:	litres
- N° lot solution équilibrage	:	
- pH VIII + sels	:	
- Osmolarité VIII + sels	:	mosm/l
- **Prise de l'échantillon 3 85 4**	:	
- Volume de FVIII à injecter	:	

	3 08 0	3 08 1
- **Prise de l'échantillon 3 08 0 et 3 08 1**	:	.....
- pH de sortie colonne	:	.....
- Osmolarité de sortie colonne	:	..... mosm/l

- Heure d'injection du VIII	: .....H.....	mn
- Débit d'injection du VIII	:	l/h
- Heure fin injection du VIII	: .....H.....	mn
- **Prise de l'échantillon 3 86 0**	:	

FRAC 03 / 02

PRODUCTION SUPER VIII THP

DATE : .....

LOT : .....

-2- Déroulement de la chromatographie

-b- Injection du prélavage

- Heure d'injection du prélavage : .....H..... mn
- Débit d'injection du prélavage : ..... l/h
- Volume de prélavage recueilli : .....
- **Prise de l'échantillon 3 86 1** : .....

-c- Injection de l'élution

- Heure d'injection de l'élution : .....H..... mn
- Débit d'injection de l'élution : ..... l/h
- Heure de fin de chromatographie : .....H..... mn

- **Volume de FVIII recueilli** : ..... **litres**
- **Prise de l'échantillon 3 85 5** : .....

-VI- FILTRATION STERILISANTE :

- Séquence PALL → SLK 7002 NFP
- Numéro de lot d'albumine → 500 ml AB 4% : with saturation with albumin.
- Numero d'autoclave : → Millipore CVGL - Durapo without albumin.
  - Filtre : .....
  - Flacons : .....

- **Volume de Facteur VIII après filtration** : ..... **Litres**

VISAS DES AGENTS DE PRODUCTION :

-
-
-
-

APPENDIX 8.3

Freeze Drying Cycle

C:/WS5/REPORTS/FOSTER/KG070016.090

CENTRE DE TRANSFUSION SANGUINE DE LILL

CONFIDENTIEL

DATE ..... : 10-07-1990
RECETTE N° : 9
N° DE CUVE :

HEURE ..... : 12:04:03
TITRE ..... : SVIIITHP
N° de LOT :
N° de LOT :
N° de LOT :

AUCUNE INACTIVATION REALISEE A CE JOUR

PREREFROIDISSEMENT DES ETAGERES : N

CONGELATION

REGULATION DE TEMPERATURE DES ETAGERES

	temperature finale	duree de la rampe	duree du palier
RAMPE 1	-58	10	240
RAMPE 2	-48	60	180
RAMPE 3	-58	60	10

TEMPERATURE DE CONGELATION DU PRODUIT ..... : -50 °C
DUREE DE MAINTIEN DU PRODUIT A BASSE TEMPERATURE : 30 mn

MISE SOUS VIDE

PRESSION P1 ..... : 140
PRESSION P2 ..... : 75
DUREE DE MAINTIEN SOUS VIDE : 90 mn

SUBLIMATION

REGULATION DE TEMPERATURE DES ETAGERES

	temperature finale	duree de la rampe	duree du palier
RAMPE 1	0	240	3840
RAMPE 2	4	960	960

TEMPERATURE PRODUIT A NE PAS DEPASSER ..... : -40 °C
DUREE DE SURVEILLANCE DE CETTE T°C DEPUIS LE DEBUT DE LA SUBLIMATION : 240 mn
TEMPERATURE DU PRODUIT DECLENCHANT LA DESSICATION SECONDAIRE ..... : 2°C
CAPACITE REDUITE SUR LE CONDENSEUR ..... :

REGULATION DE PRESSION CUVE

	pression	duree du palier
PALIER 1	55	6000

CENTRE DE TRANSFUSION SANGUINE DE LILL

CONFIDENTIEL

DATE ..... : 10-07-1990
RECETTE N° : 9
N° DE CUVE :

HEURE ..... : 12:04:03
TITRE ..... : SVIIITHP
N° de LOT :
N° de LOT :
N° de LOT :

AUCUNE INACTIVATION REALISEE A CE JOUR

PREREFROIDISSEMENT DES ETAGERES : N

CONGELATION

REGULATION DE TEMPERATURE DES ETAGERES

	temperature finale	duree de la rampe	duree du palier
RAMPE 1	-58	10	240
RAMPE 2	-48	60	180
RAMPE 3	-58	60	10

TEMPERATURE DE CONGELATION DU PRODUIT ..... : -50 °C
DUREE DE MAINTIEN DU PRODUIT A BASSE TEMPERATURE : 30 mn

MISE SOUS VIDE

PRESSION P1 ..... : 140
PRESSION P2 ..... : 75
DUREE DE MAINTIEN SOUS VIDE : 90 mn

SUBLIMATION

REGULATION DE TEMPERATURE DES ETAGERES

	temperature finale	duree de la rampe	duree du palier
RAMPE 1	0	240	3840
RAMPE 2	4	960	960

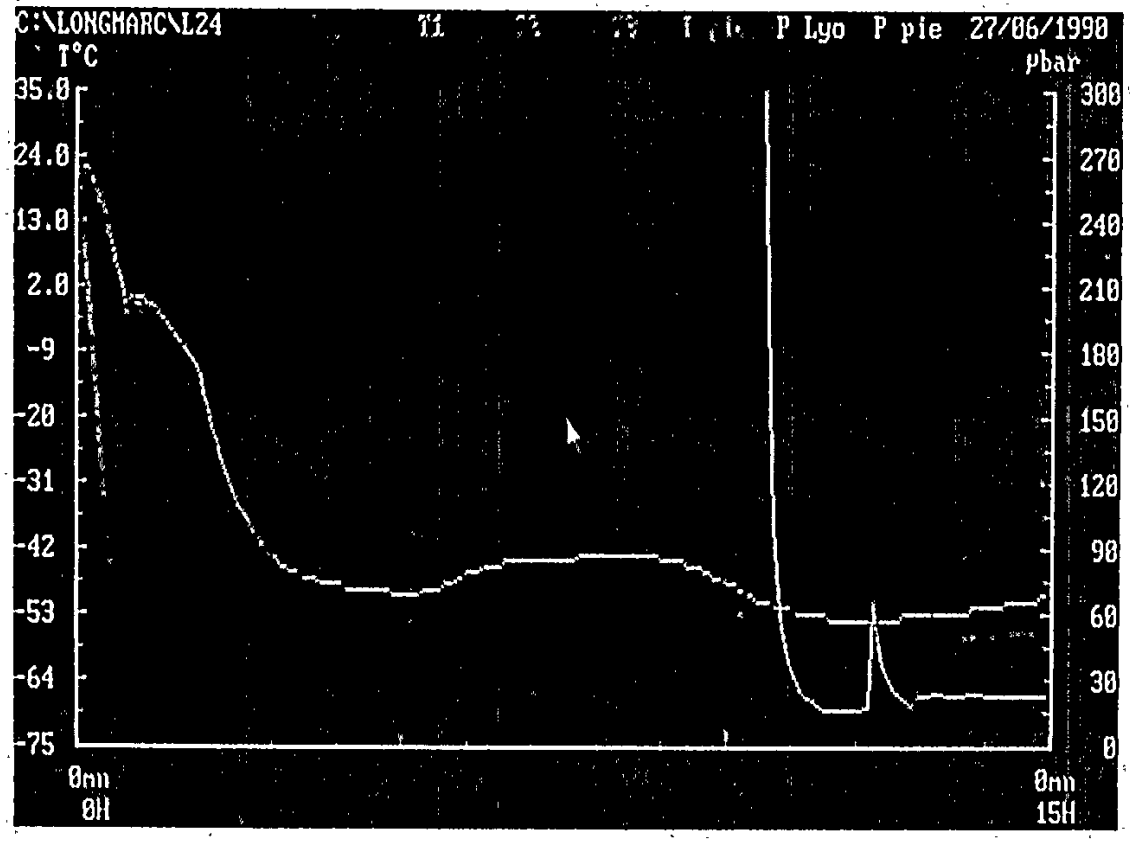
TEMPERATURE PRODUIT A NE PAS DEPASSER ..... : -40 °C
DUREE DE SURVEILLANCE DE CETTE T°C DEPUIS LE DEBUT DE LA SUBLIMATION : 240 m
TEMPERATURE DU PRODUIT DECLENCHANT LA DESSICATION SECONDAIRE ..... : 2°C
CAPACITE REDUITE SUR LE CONDENSEUR ..... :

REGULATION DE PRESSION CUVE

	pression	duree du palier
PALIER 1	55	6000

CONFIDENTIEL

Freezing stage.



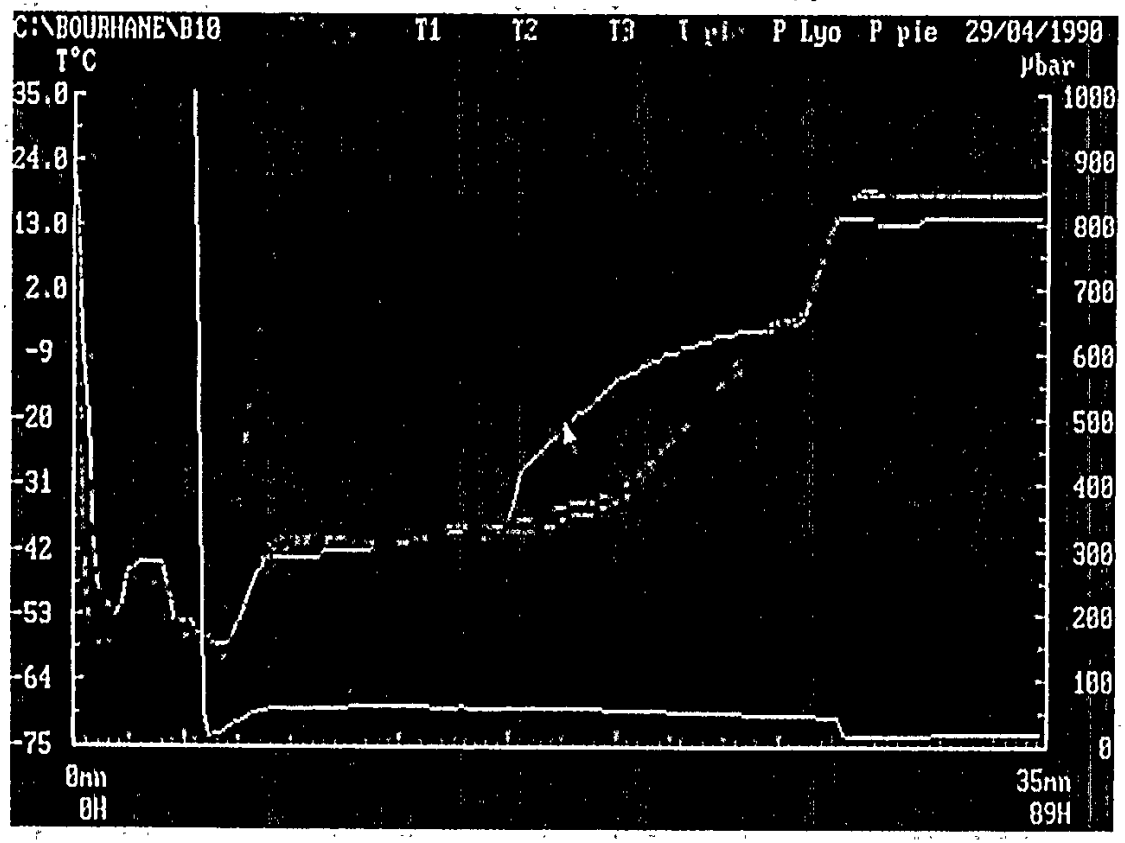
↑ Supercooling

↑ Temperature

FVM Drying Cycle (10 ml / 111)

CONFIDENTIEL

SJIII THP 10

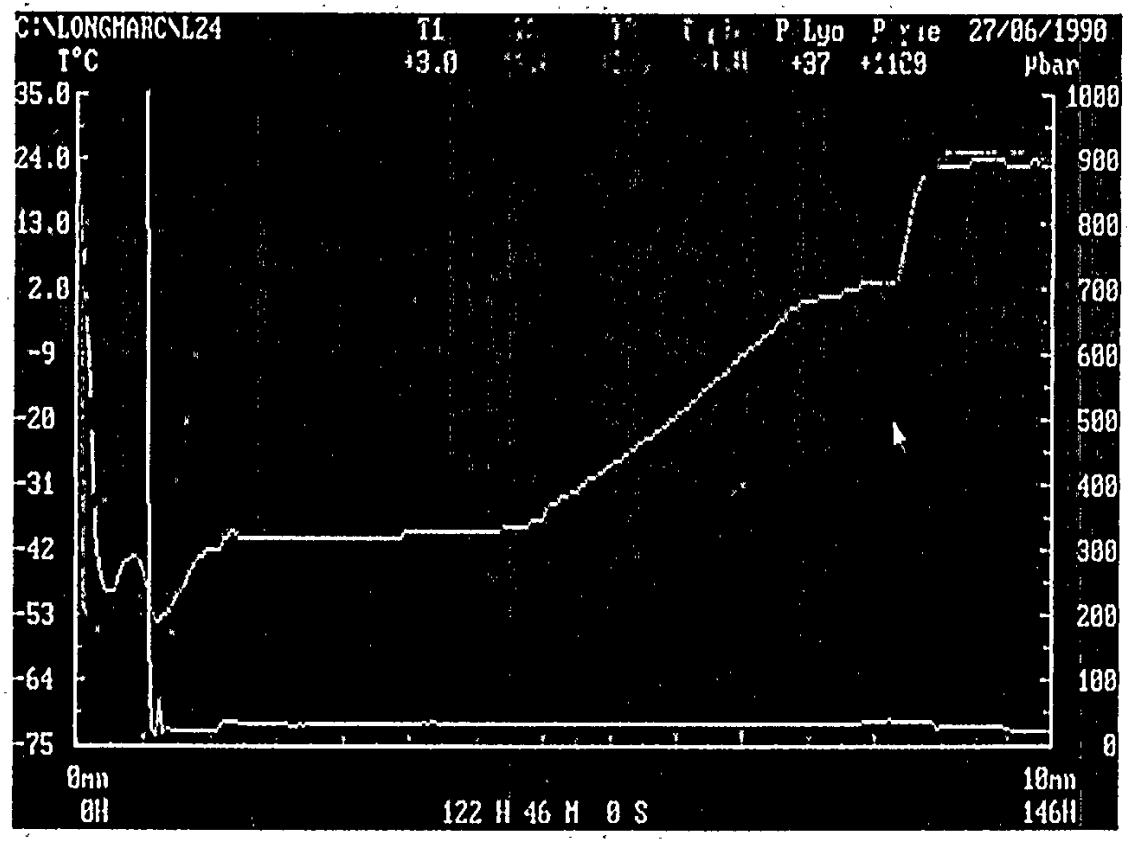


Freezing | Primary Drying | Secondary Drying

CONFIDENTIEL

PVB Drying Cycle (20 ml fill)

S VIII THP EOHL.



SNB.007.7368

APPENDIX 8.4

Analytical Methods TNBP
Tween
Moisture

C:/WS5/REPORTS/FOSTER/KG070016.090

PROCÉDURE ☐ GÉNÉRALE ☐ SPÉCIFIQUE	PROCEDURE N° FT 74 SERVICE CONTROLE QUALITE
TITRE : TNBP CONTENT	DATES : Original issue : MAY 6 Previous issue : DEC. 87 This issue : JUNE 90 Revision : 2

DESTINATAIRES : EXEMPLAIRE N° :

PRINCIPLE

The residual Tri-n-butyl phosphate (TNBP) content is determined by gas chromatography. SD samples are extracted and analysed to measure their TNBP content after comparison with a standard curve.

PROCEDURE

1) Extraction

- Introduce 3 ml of sample into 15 ml tube with a screw cap and a teflon liner for centrifuging.

- Add 5 µl of octanoic acid (internal standard concentrated at 5µg/ml or 5000 ppm in hexane).

- Add 2.5 g of Na_2So_4

- Add 1ml of ethanol at 95% V/V

- Add 0.5 ml of 1.5 N perchloric acid, cap the tubes and use Vortex

- Warm in a waterbath for 10 mn at 37°C

- Add 4 ml of hexane, recap and shake vigorously to produce an emulsion.

- Centrifuge the samples (10 °C, 2,000 r.p.m., 30 minutes).

- In taking great care not to disturb the interface, place the organic phase in a 5 ml centrifuge tube.

- Concentrate the extract under a N_2 stream in ice. Improves Recovery of C.

Important: Do not overdry the samples. Rinse the walls of the tubes with 100 to 200 µl of hexane to collect the whole product. Then concentrate it in a tube. Evaporate hexane under a N_2 stream.

- Dissolve the sample in 10 µl of hexane and then analyse it..

2) Analysis by Gas chromatography

- PERKIN - ELMER GAS CHROMATOGRAPH 8 500

- Use a 140 cm x 6 mm x 2 mm glass columm packed with 10% SP-1000 or 80/100 mesh Supelcoport.

- Chromatography conditions : isothermal without sample derivatization.

- Integrator : SHIMADZU CHROMATOPAC C-R3A.

- Sample for injection : - 1 µl of the tested sample
  - standard solutions which contain a known amount of TNBP.

Validés by spiking with TNBP ≥ 0.1 ppm

1 ~ 2 ppm typical FVIII

10 ppm FDA limit

APPROVAL : date : SIGNATURE

PROCÉDURE ☐ GÉNÉRALE ☐ SPÉCIFIQUE	PROCEDURE N° FT 76 SERVICE CONTROLE QUALITE
TITRE : ASSAY OF TWEEN 80	DATES : Original issue : FEV. 88 Previous issue : FEV. 88 This issue : JUNE 90 Revision : 1

DESTINATAIRES : EXEMPLAIRE N° :

PRINCIPLE

Colorimetric assay following extraction of TWEEN 80.

- Alkaline hydrolysis of the samples
- Neutralization
- Extraction of TWEEN 80 with methanol
- Formation of a TWEEN 80 barium iodide colour complex.

EXPERIMENTAL METHOD

Material :

Glass tube with teflon lined caps

Reagents

- 50 % (W/W) sodium hydroxide
- 5 % (W/V) barium chloride in 1N hydrochloric acid
- 4.5N hydrochloric acid
- N/10 iodine solution (MERCK)
- 0.1% (W/V) TWEEN 80 solution

PROCEDURE

Standard and sample preparation

FDA
≥ 10ppm ≤ 100ppm

Dilute the samples to 1% of protein.
Prepare a 0.1% (W/V) TWEEN 80 solution

Prepare a standard curve : to 500 µl of the sample add varying amounts of 0.1% TWEEN 80 (from 0 µl to 50µl). Then add sufficient diltilled water to produce 700µl.

Prepare a water blank (700µl) and a protein blank using 500µl of 1% albumin and 200 µl of distilled water.

Test : 500 µl of sample containing 1% of protein and 200 µl of distilled water.

Alkaline hydrolysis

To each sample add 0.3 ml of 50% sodium hydroxide.
Tightly cap the tubes and put them into a waterbath (95°) for at least 12 hours.

Neutralization

To each hydrolyzed sample add 2 ml of 4.5 N hydrochloric acid.
Centrifuge and collect the clear surpernatant in a tube.

Extraction

Add 2 ml of methanol to the deposit, shake vigorously and centrifuge again. Transfer the supernatant into the relating tubes.

Therefore each tube contains 5 ml of solution.

Note : The methanol extraction can be carried out after colour development, if a precipitate is formed. Methanol is a good solvent for TWEEN 80 and TWEEN 80-BARIUM-IODIDE complex.

Formation of the coloured complex

In each tube introduce 0.75 ml of barium chloride.
- Add 0.37 ml of N/10 iodin and mix
- Wait 15 mn and measure the optical density (OD) at 535nm.
- Use the water treated blank to zero the spetrophotometer.
  Subtract the optical density of the protein blank from all readings of the standard.
- Plot the OD straight line : point on the the x-axis the TWEEN 80 concentration .
- The TWEEN 80 concentration is the intersection point of the sample straight line with the X-axis.

APPROVAL : date : SIGNATURE

PROCEDURE N° FT 07/0
SERVICE CONTROLE QUALITE

PROCEDURE		DATES	
☐	générale		Première édition : 28.02.1990
			Précédente édition:
			Cette édition : 28.02.1990
☐	spécifique		Révision : 0

DETERMINATION OF RESIDUAL MOISTURE

Principle

Titration of water with the KARL FISCHER reagent and determination of the equivalent point by means of an ammeter.

Apparatus

- BECKMAN aquameter KF 4B
- METTLER balance (1/10 mg.)
- Dry recipients for titration with the KARL FISCHER reagent

Standardisation

- Introduce 40 ml. of methyl alcohol into a KARL FISCHER recipient and titrate with the reagent (V_1 ml.).
- In another container place an accurately weighed quantity of sodium tartrate (containing 15.66 per cent of water) equivalent to about 100 mg. and 40 ml. of methyl alcohol and titrate with the reagent (V_2 ml.).

$$\text{Titre: } T = \frac{15.66 \times 100}{P_{mg} \times (V_2 - V_1)} = \text{mg. of water per millilitre}$$

P = weight of sodium tartrate

Test

Use the method employed for the standardisation (V_1 ml for the blank and V_3 ml for the sample).

$$\text{Residual moisture: } \frac{(V_3 - V_1) \times T \times 100}{P_1} = (\%)$$

P_1 = weight of the lyophilisate (about 200 mg.)

Limit: ≤ 2 %

APPROBATION le : 17 MARS 1990

B. FLAN
Contrôle Qualité

Single repetitive dry transfer with base. Rinse as quickly as possible in dilute NaOH with prolonged stirring to dispose

1, 0.5 – 1.0% Max

0.3% – 0.8% Typical

Eff. Max 2%

APPENDIX 8.5

Clinical data used to validate VIII C assay methodology

(i) Table of detailed clinical and assay data

(ii) Full manuscript (with modified table 2)

Table 2

Individual recoveries (%) calculated on the basis of the FVIII:C levels, measured by the one-stage (FVIII:C-1), two-stage (FVIII:C-2) or chromogenic (FVIII:C-c) assays, in the HP,S/D FVIII concentrate infused and in 1 hr post-infusion plasma samples of 5 severe haemophilia A patients.

Patient number	Batch infused	Method of determination of FVIII concentrate potency	Method of determination of FVIII:C plasma levels		
			FVIII:C-1	FVIII:C-2	FVIII:C-c
1	A	FVIII:C-1[a]	96.0	59.0	-
		FVIII:C-2[a]	111.1	68.4	-
2	B	FVIII:C-1[a]	119.2	99.3	90.8
		FVIII:C-1[b]	96.0	80.0	73.1
		FVIII:C-2[a]	132.7	110.5	101.1
3	C	FVIII:C-1[a]	97.4	75.3	83.1
		FVIII:C-1[b]	84.0	64.9	71.7
		FVIII:C-c[a]	88.5	68.5	75.6
		FVIII:C-c[b]	87.9	68	75
4	C	FVIII:C-1[a]	116	95.7	79.8
		FVIII:C-1[b]	100	82.5	68.8
		FVIII:C-c[a]	105.5	87	72.5
		FVIII:C-c[b]	104.7	86.4	72.1
1	D	FVIII:C-1[b]	70.5	59.1	59.1
		FVIII:C-c[b]	82.4	69.0	69.0
5	E	FVIII:C-1[b]	96.7	81.9	63.1
		FVIII:C-c[b]	126.9	107.5	82.8

[a] = procedure 1 (predilution in buffer, concentrate standard)
[b] = procedure 2 (predilution in FVIII-deficient plasma, plasma standard)

Thr. Haem. : —

207[1] - - ' '

VALIDATION OF A PROCEDURE FOR POTENCY ASSESSING OF A HIGH PURITY FACTOR VIII CONCENTRATE. COMPARISON OF DIFFERENT FACTOR VIII COAGULANT ASSAYS AND EFFECT OF PREDILUENT

Authors : Claudine Mazurier, Armelle Parquet-Gernez, Maurice Goudemand.

Running title : Assessment of potency of a high-purity FVIII concentrate.

Institute : Centre Régional de Transfusion Sanguine de Lille (Directeur : Dr J.J. Huart), France.

Correspondence to : Dr C. Mazurier, laboratoire d'Hémostase, C.R.T.S. Lille, 21 rue Camille Guérin, 59012 LILLE Cédex France.

SUMMARY

The assessment of factor VIII coagulant activity (FVIII:C) in recently available highly purified and concentrated FVIII therapeutic products calls for careful evaluation of assay methodologies. We assayed more than 130 batches of a concentrate with a specific activity of about 150 FVIII:C units/mg protein, using one-stage and two-stage clotting and chromogenic methods.There was good agreement between the potency estimates obtained with the different methods. We also compared the FVIII:C potencies obtained after predilution in buffer or FVIII-deficient plasma using either calibrated plasma or FVIII concentrate as references. With the one-stage assay we found a marked discrepancy between the potency values obtained with buffer and with FVIII- deficient plasma used as prediluents. In order to validate our "in vitro" data we performed 6 "in vivo" analyses in severe haemophilia A patients. On the basis of the overall data obtained we chose to label FVIII potency by using FVIII-deficient plasma as prediluent, reference plasma as standard and the chromogenic assay method.

KEY WORDS

FVIII:C assay, FVIII concentrate.

INTRODUCTION

The measurement of the potency of factor VIII (FVIII) concentrates is an important problem of haemophilia A therapy. Indeed, the potency assigned to a batch is the crucial parameter determining as a function of the patient's plasma volume the FVIII coagulant (FVIII:C) level expected after infusion. As the three techniques of FVIII:C assay, i.e. one-stage (1,2), two-stage clotting (3) and chromogenic (4,5) assay, were in fact set up to measure plasma FVIII:C levels, the FVIII:C levels assigned to a concentrate are tainted by an error proportional to the coefficient of variation (CV) of the technique used and the magnitude of the dilution needed to adjust concentrate FVIII:C level to that in normal plasma. Consequently, one of the causes of inadequate potency is related to the FVIII concentration of the product. Another is related to the biological activity of purified FVIII molecules, particularly the "state of activation" and the stability of FVIII which may vary from one product to another, according to their purity and purification process and are different from native FVIII in plasma milieu.

These FVIII assay problems are made worse by the recently available and so-called very high purity (VHP) FVIII concentrates (specific activity > 1000 IU FVIII:C/mg proteins before addition of stabilizing albumin), as recently pointed out in a collaborative study set up by T. Barrowcliffe (N.I.B.S.C., London) to investigate inter-laboratory and inter-methods variability in assays of VHP and intermediate-purity (IP) concentrates (6) and by Dawson et al. (7). To our knowledge, these authors are the only ones who have produced a study with a limited number of FVIII:C assays on high purity (HP, specific activity : 50 - 300 IU

FVIII:C/mg proteins) or VHP concentrates. Our paper deals with our 18 months-long experience, covering more than 250 assays, acquired in monitoring the potency of an HP concentrate and adapting our assay methods to ensure more accurate FVIII:C assigment of each batch.

MATERIALS AND METHODS

FVIII concentrate

The FVIII concentrate studied (HP.S/D FVIII concentrate) was prepared by the fractionation department of C.R.T.S. Lille and is distributed by Biotransfusion (France). It was prepared from cryoprecipitate by a procedure including aluminium hydroxide adsorption, cold precipitation, treatment by solvent and detergent (S/D) for viral inactivation and anion exchange chromatography. The final product was freeze-dried without addition of serum albumin as a stabiliser and its main characteristics established on the 71 batches produced in 1988 were as follows : FVIII:C = 33.8 ± 9.1 IU/ml, FVIII:Ag = 48.6 ± 15.9 IU/ml, fibrinogen = 0.06 ± 0.05 g/l, proteins = 0.22 ± 0.08 g/l, specific activity = 164 ± 56.4 IU FVIII:C/mg proteins.

Prediluents

Buffer : 27 mM sodium acetate-barbital and 120 mM NaCl adjusted to pH 7.4 with HCl (Michaelis buffer).

Natural FVIII-deficient plasma : frozen plasma from a severe haemophilia patient whose blood was drawn on 1/10 x volume 0.13 M trisodium citrate.

Artificial FVIII-deficient plasma : was prepared by treating, with EDTA, the plasma from normal blood collected on ACD according to a previously published procedure (8). This reagent contains < 1 U/dl of FVIII, > 50 U/dl of factor V and von Willebrand factor (vWF) and normal levels of fibrinogen and of other coagulation factors. It was lyophilised in 2 ml aliquots.

Standards :

The two lyophilised in-house standards used were prepared in C.R.T.S. Lille and calibrated against the following international standards (N.I.B.S.C., London) kindly provided by Dr T. Barrowcliffe : 1st international reference preparation 80/511 for factor VIII-related activities in plasma and 3rd international standard, human concentrate 80/556, for factor VIII:C. The plasma reference 7030 was prepared in August 87. After reconstitution with 2 ml H_2O it was shown to contain 1.1 IU FVIII:C/ml. The concentrate reference 7001 is an intermediate-purity product (SA = 1 UI FVIII:C/mg) distributed in 1 ml aliquots and lyophilised in January 88. On the basis of the results obtained from 4 European laboratories involved in quality control of FVIII concentrates, it was assumed to contain 21.5 IU FVIII:C/ml after reconstitution with 1 ml H_2O.

FVIII:C assays

The freshly reconstituted concentrates to be tested were prediluted just before the different assays in either buffer or FVIII-deficient plasma in order to adjust FVIII:C level to approximately 1 IU/ml. Three to 5 working dilutions kept on ice were then made from 2 separate prediluted (1/40) samples.

In the one-stage assay technique (2) we used 5 working dilutions (1/20, 1/40, 1/80, 1/160, 1/320) in Michaelis buffer of the reference (either plasma reference 7030 or concentrate reference 7001 prediluted 1/20) and of the prediluted concentrate to be tested. The assay was semi-automated, using a KC 10 (Amelung) instrument to determine the clotting time end point. Each test was performed with a 50 µl diluted sample to which 50 µl natural FVIII-deficient plasma was added. After 1 min incubation at 37°C, 50 µl of a suspension of 1 vol 10 g/l kaolin and 1 vol of a 1/25 dilution of cephalin (Stago^{R}) was added ; then 50 µl of 25mM $CaCl_2$ prewarmed to 37°C was added after 8 min of activation at 37°C. FVIII:C data (FVIII:C-1) were calculated on calibration curves drawn on semilog paper.

Two-stage assay was performed according to the published method (3) taking care to avoid aluminium hydroxide adsorption of FVIII concentrate samples. The assay technique used 5 working dilutions (1/40, 1/80, 1/160, 1/320, 1/640). To 0.3 ml of a fresly reconstituted lyophilised reagent (1 Vol 1/50 dilution of cephalin (Stago^{R}), 1 Vol 1/20 dilution of bovine factor V (Diagen^{R}) and 1 Vol of 1/10 diluted human serum (the contact factors of which were activated by 6 hrs incubation at 4 °C in a glass tube), 0.1 ml of diluted sample and, after 1 min incubation at 37°C, 0.1 ml of prewarmed 25mM $CaCl_2$ were added. The optimum time for factor Xa generation from adsorbed plasma or diluted samples of non adsorbed concentrates was previously determined and ranged from 15 to 20 min. A 0.1 ml aliquot of the incubated mixture was then added, with 0.2 ml of a pool of citrated normal plasma, to 0.2 ml of prewarmed 25mM $CaCl_2$. The clotting time was then determined manually. FVIII:C data (FVIII:C-2) were calculated from log-log established calibration curves.

Chromogenic assay was performed with a kinetic microplate technique at room temperature using the "Chromo FVIII:C" kit (Stago^{R}). Predilutions and working dilutions were made in the buffer, containing 1 % bovine serum albumin, available in this kit. Five working duplicate dilutions of reference (1/20, 1/30, 1/40, 1/60, 1/80), a blank (buffer) and 3 duplicate dilutions (1/30, 1/40, 1/60) of prediluted concentrates were used. In each well test, 40 µl of diluted sample, 40 µl of reagent 1 (factor IXa + phospholipids) and, after 4 min incubation, 40 µl of reagent 2 (factor X + $CaCl_2$) were added. After 10 min incubation, 40 µl of CBS 4803 substrate were added and the optical density (OD) was measured with a Vmax microplate reader (Molecular Device). FVIII:C data (FVIII:C-c) were calculated from the calibration curves established with a connected computer and a program ("Softmax", Molecular Device) based on linear or quadratic functions established between the FVIII:C levels of the dilutions of standard and their Δ OD/min.

"In vivo" study

All severe adult haemophilia A patients gave their informed consent for "in vivo" evaluation : they received a single infusion of a dose of $\simeq$ 30 IU FVIII:C/kg body weight (b.w.). Blood samples were collected in 0.13 M trisodium citrate before and 1 hr after infusion. Citrated plasma was prepared by centrifugation at 2000 g for 20 min and stored frozen at - 35°C until studied in the week following infusion. All pre- and post-infusion FVIII:C levels were assayed using buffer as prediluent and the plasma reference 7030, calibrated against the 1st international preparation 80/511, as standard. FVIII:C recoveries were calculated on values obtained 1 h after infusion and expected values based on a theoritical 43.3 ml/kg

plasma volume.

RESULTS

Correlation between the different techniques (procedure 1)

A total of 42 batches of HP, S/D FVIII concentrate was analyzed in parallel by one-stage and two-stage assays, using buffer as prediluent and the reference concentrate 7001 as standard (procedure 1). The analysis of the data obtained gave the following regression line (Fig.1A) : FVIII:C-2 = 0.82 x FVIII:C-1 + 2.2 with a correlation coefficient, r = 0.87, the one-stage method producing on average 13.1 % more (Table 1). Twenty six of these batches were also analyzed by chromogenic assay. The correlation coefficient determined by linear regression analysis between FVIII:C-2 and FVIII:C-c data was 0.80. The precision of the two assay methods based on FXa generation was estimated by calculating the geometric coefficients of variation (%) of the data obtained in 2 assay series on 10 lyophilized 2 ml aliquotes of a batch of HP,S/D FVIII concentrate. Significantly less assay variability was observed with the chromogenic assay (CV = 4.0 %) than with the two-stage assay (CV = 9.9 %). On account of its greater precision and accuracy and its capacity to adapt to a larger number of samples, we switched to chromogenic assay in July 88, leaving two-stage assay for routine quality control of FVIII concentrate production. The results of chromogenic assays also showed good correlation with those of one-stage assays (n = 52 ; r = 0.83 ; FVIII:C-c = 0.77 x FVIII:C-1 + 4.7 (Fig.1B) and the absolute potencies differed on average by 9.7 % (Table 1).

Effect of prediluent (comparison of procedures 1 and 2)

Procedure 2 consists in prediluting FVIII concentrate in FVIII deficient plasma. Due to the FVIII concentration of HP.S/D concentrate (34 ± 9 U/ml), this means that concentrate is diluted 1/40 to reach plasma FVIII level. Consequently we decided to use as standard the plasma reference 7030.

The effect of prediluent was analyzed on 14 batches with one-stage assay. Shorter APTT values were always obtained when FVIII concentrate was prediluted in FVIII-deficient plasma. Similar APTT values were found when FVIII concentrates were prediluted in either artificial or natural FVIII-deficient plasma. The APTT values were converted to FVIII:C level by using the calibration curves established with reference concentrate 7001 (procedure 1) or reference plasma 7030 (procedure 2). We observed varying differences (30 ± 25 %) in the FVIII:C-1 content determined with the 2 procedures : FVIII:C-1 (procedure 2) = 0.42 x FVIII:C-1 (procedure 1) + 29.3 with very poor correlation (r = 0.33) between the results obtained with the two procedures, but always higher values when using FVIII-deficient plasma for predilution (Fig.2A).

The effect of predilution was analyzed with chromogenic assay on a total of 22 batches (the 14 mentioned above and 7 others). The analysis of the data obtained gave the following regression line : FVIII:C-c (procedure 2) = 1.19 x FVIII:C-c (procedure 1) - 0.16 with a very good correlation coefficient (r = 0.92) (Fig.2B) between the results obtained with the two procedures. The results obtained with procedure 2 using one-stage and chromogenic assays were compared in a total of 39 batches : FVIII:C-c = 0.93 x FVIII:C-1 + 1.55. The correlation coefficient (r = 0.89) and the slope of the regression line indicate that the results were well correlated

but the one-stage assay produced on average of 15.7 % more (Table 1).

"In vivo" study

To validate our data we carried out 6 "in vivo" analyses in 5 severe haemophilia A patients and the results are summarized in Table 2. The calculated recovery values varied according the different methods of determination of FVIII concentrates potency and of plasma assessment. The recoveries calculated on the basis of FVIII:C plasma assessments made with the one-stage method ranged between 70.5 and 132.7 % (n = 17, mean ± SD = 100.9 ± 16.5 %) according to the different methods used for the determination of FVIII concentrate potency. The recoveries obtained when either the two-stage assay (n= 17, mean ± SD = 80.2 ± 15.9 %) or the chromogenic assay (n = 15, mean ± SD = 75.8 ± 10.6 %) was used for plasma FVIII:C assessment were 14.6 - 38.4 % lower than those obtained with one-stage assay. As far as the procedure used to determine FVIII concentrate potency is concerned, we obtained recovery values ranging from 59 to 132.7 % with procedure 1 (n = 20, mean ± SD=92.4 ± 18.5 %) and ranging from 59.1 to 126. 9 % with procedure 2 (n = 29, mean ± SD= 80.9 ± 16.2 %).

DISCUSSION

Discrepancies between one-stage and two-stage FVIII assays in FVIII concentrates have been the subject of several papers (9-12). The use of standard concentrate has been recommended to reduce this problem (11, 13, 14), but has not resolved it, particularly when testing highly purified FVIII concentrates (7). As for the last 18 months we have been dealing with the quality control of the FVIII:C potency of a FVIII concentrate with a specific activity ca. 150 IU FVIII:C/mg, we thought it useful to report our data about comparative results obtained using 2 types of assay techniques (one-stage and two-stage either clotting or chromogenic assays), 2 prediluents (buffer and FVIII-deficient plasma) and 2 standards (concentrate and plasma references).

The comparative results obtained with one-stage, two-stage and chromogenic assays have shown that they are generally well correlated. Nevertheless, overall higher FVIII:C-1 values were found and are assumed to be due to some degree of activation of FVIII during the purification process.

Initial results obtained with one-stage assay have shown that the diluting medium influences test results. Shorter clotting times were always obtained when concentrate was diluted in FVIII-deficient plasma instead of buffer. This was in agreement with previously published results (15, 16). However, the differences between the values varied between 2.7 and 85.7 % (30 ± 25 %). Moreover, the lack of correlation between the data obtained with the two prediluents supports the notion that the "FVIII:C enhancing effect" of FVIII-deficient plasma varies from one batch of concentrate to another. It would be interesting to study this effect to see

if it is related to the amount of activated FVIII present in the different batches. On the other hand, the results obtained with chromogenic assay provided evidence that the differences between the potency measured after predilution with buffer and FVIII-deficient plasma varied only between 0.7 and 30.5 % (17 ± 11 %). Moreover, good correlation was found between the values obtained with the two prediluents. This shows that the diluting medium has much less influence on chromogenic assay that on one-stage assay and that this influence is more stable from one batch to another when using chromogenic assay. This may be explained by the presence of 1.5 ‰ (w/v) albumin in the buffer used for chromogenic assay and/or by the fact that chromogenic assay, like two-stage clotting assay, is not sensitive to activated FVIII. It would be interesting to see if the incorporation of such a concentration of albumin in one-stage and two-stage assays also improved the results.

An "in vivo" study was performed to validate our "in vitro" FVIII:C assays. Although we cannot rule out the possibility we have underestimated recovery values in the event of the FVIII peak occurring earlier than 1 hr post-infusion (17), we can draw the following conclusions: we first confirmed that the recovery values were always lower when using two-stage assays to estimate FVIII:C levels of 1 hr post-infusion samples (18). We also noticed some differences between recovery values according to the method used for assessing FVIII concentrate potency ; however, given the large interindividual variability of FVIII:C recoveries (18-23), the number of patients we studied was much too small to draw any conclusion about the superiority of a given assay or procedure. Nevertheless, it was sufficient to show that if labelled potency was obtained with one-stage assay and procedure 2 and if replacement therapy was controlled with

chromogenic assay, rather deceptive 1 hr post-infusion recoveries (67.2 ± 5.9 %) could result. On the other hand, calculating recoveries in 4 patients infused with 3 different batches of the product on the basis of FVIII concentrate potencies determined with chromogenic assay and procedure 2 gave satisfactory values (86.0 ± 18.4 %) ranging from 68 to 127 % according to the method used to assess post-infusion FVIII:C plasma level.

That is why we chose to determine the FVIII potency of each batch of HP, S/D concentrate on the basis of the FVIII:C data obtained in prediluting it in FVIII-deficient plasma, using a plasma reference and the chromogenic assay technique (FVIII:C-c, procedure 2). Indeed, the results reported here have shown that this method is accurate : it is reproducible (CV = 4 %), well correlated with one-stage assay (r = 0.89) and validated by "in vivo" recovery values.

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REFERENCES

1. LANGDELL RD, WAGNER RH, BRINKHOUS KM. Effect of antihaemophilic factor on one-stage clotting tests. A presumptive test for hemophilia and a simple one-stage antihemophilic assay procedure. J Lab Clin Med 1953 ; 41:637-647.

2. SOULIER JP, LARRIEU MJ. Nouvelle méthode de diagnostic de l'hémophilie. Dosage des facteurs antihémophiliques A et B. Sang 1953 ; 24:205-215.

3. BIGGS R, EVELING J, RICHARDS G. The assay of antihemophilic globulin activity. Br J Haematol 1955 ; 1:20-34.

4. SEGHATCHIAN MJ, MILLER-ANDERSON M. A colorimetric evaluation of factor VIII:C potency. Med Lab Sci 1978 ; 35:347-354.

5. ROSEN S. Assay of factor VIII:C with a chromogenic substrate. Scand J Haematol 1984 ; 33 (supp 40):139-145.

6. NIBSC unpublished report, 1989.

7. DAWSON NJ, KEMBALL-COOK G, BARROWCLIFFE TW. Assay discrepancies with highly purified factor VIII concentrates. Haemostasis 1989 ; 19:131-137.

8. CHANTARANGKUL V, INGRAM GIC, THORN MB, DARBY SC. An artificial haemophilic plasma for one-stage factor VIII assay. Br J Haemat 1978 ; 40:471-488.

9. KIRKWOOD TBL, BARROWCLIFFE TW. Discrepancy between one-stage and two-stage assay of FVIII:C. Br J Haematol 1978 ; 40:333-338.

10. BARROWCLIFFE TW, THOMAS DP. Factor VIII standardization. Lancet 1981 ; ii:1342.

11. BARROWCLIFFE TW. Comparisons of one-stage and two-stage assays of factor VIII:C. Scand J Haematol 1984 ; 33 (supp 41):39-54.

12. MIKAELSSON M, OSWALDSSON U. Standardization of VIII:C assays : a manufacturer's view. Scand J Haemat 1984 ; 33 (supp 41):79-86.

13. BARROWCLIFFE TW, KIRKWOOD TBL. An international collaborative assay of factor VIII clotting activity. Thromb Haemost 1978 ; 40:270-271.

14. BARROWCLIFFE TW, CURTIS AD, THOMAS DP. Standardization of factor VIII-IV. Establishment of the 3rd international standard for factor VIII:C concentrate. Thromb Haemost 1983 ; 50:697-702.

15. LEE ML, MAGLALANG EA, KINGDON HS. An effect of predilution on potency assays of factor VIII concentrates. Thromb Res 1983 ; 30:511-519.

Table 1

Comparison of the different assay methods.

The results are expressed as the ratios (in %) of FVIII:C potencies.

Assay methods		$\frac{\text{FVIII:C-1}^a}{\text{FVIII:C-2}^a}$	$\frac{\text{FVIII:C-1}^a}{\text{FVIII:C-c}^a}$	$\frac{\text{FVIII:C-1}^b}{\text{FVIII:C-c}^b}$
Number of batches tested		42	52	39
ratios (%)	range	95 - 145	92 - 147	83 - 148
	mean	113.1	109.7	115.7
	SD	14	12	16

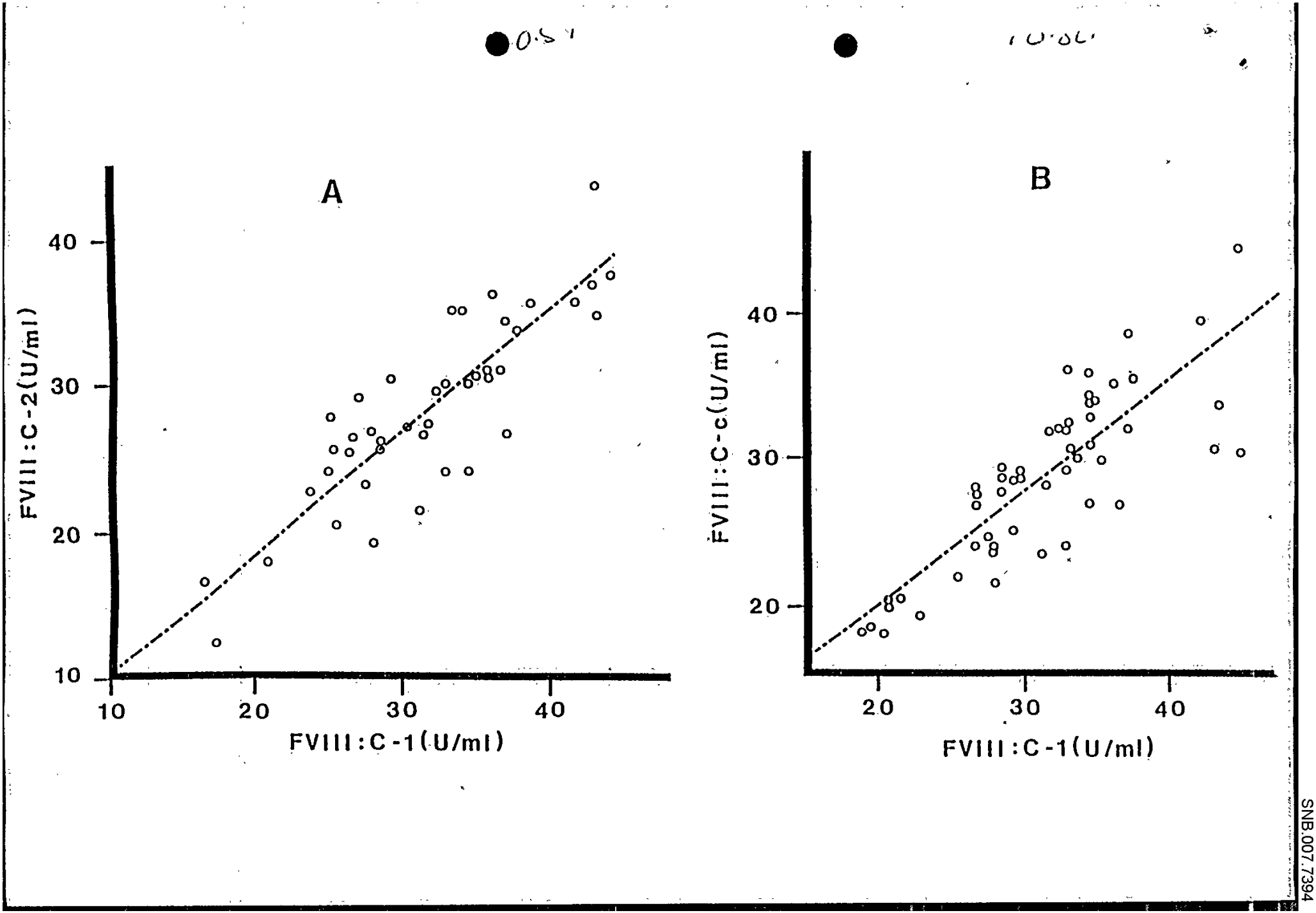
a : procedure 1 (predilution in buffer, concentrate standard)
b : procedure 2 (predilution in FVIII-deficient plasma, plasma standard)

Table 2

Recoveries obtained after 6 infusions of HP, S/D FVIII concentrate in 5 severe haemophilia A patients

Patient number	Batch infused	Method of determination of FVIII:C plasma level		
		FVIII:C-1 (range)*	FVIII:C-2 (%)**	FVIII:C-c (%)**
1	A	96.0-111.1	61.6	-
2	B	96.0-132.7	83.3	76.2
3	C	84.0-97.4	77.4	85.4
4	C	100-116	82.5	68.8
1	D	70.5-82.4	84.0	84.0
5	E	96.7-126.9	84.7	65.2

* range of the recovery values (%) obtained on the basis of FVIII concentrate potencies determined with different methods and "in vivo" FVIII: levels assayed with one-stage method.
** average values obtained on the basis of "in vivo" FVIII levels determined with two-stage and chromogenic assays, expressed as percentages of the one-stage based recovery values (*)


A
FVIII:C-2(U/ml)
40
30
20
10
10
20
30
40
FVIII:C-1(U/ml)
B
FVIII:C-c(U/ml)
40
30
20
20
30
40
FVIII:C-1(U/ml)

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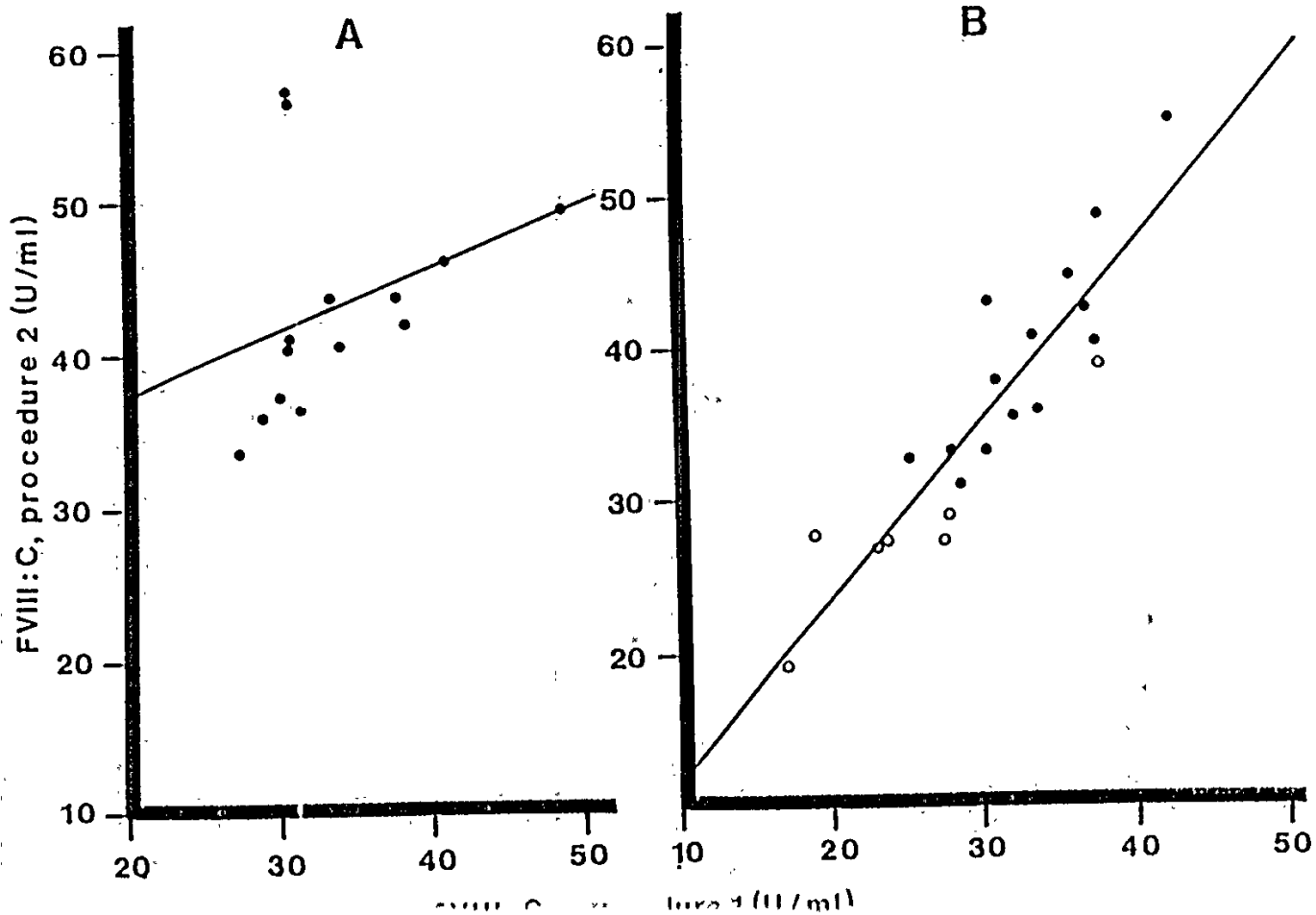
r = 0.92



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Legends to figures

Figure 1. Comparison of FVIII activity determined by one-stage method (FVIII:C-1) and either two-stage (FVIII:C-2, in A) or chromogenic (FVIII:C-c. in B) methods in different batches (⊙) of HP,S/D FVIII concentrate prediluted in buffer and calibrated against reference concentrate 7001 (procedure 1). Dotted lines represent the calculated regression lines.

Figure 2. Comparison of FVIII activity determined by procedure 1 (values in abscissa = predilution in buffer, reference concentrate 7001) and procedure 2 (values in ordinate = predilution in artificial FVIII-deficient plasma, reference plasma 7030) using one-stage (in A) and chromogenic (in B) assays. Note that the buffer purchased in the chromogenic assay kit used in B contains 1.5 ‰ bovine serum albumin. 14 batches (●) were tested in A, 7 more (⊙) were tested in B. Dotted lines represent the calculated regression lines.