V. Collaborative Study for the Establishment of Somatropin Chemical Reference Standard

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INTRODUCTION

The European Pharmacopoeia (Ph. Eur.) monograph for somatropin[1] (recombinant DNA-derived human growth hormone) no longer includes an *in vivo* bioassay. This important step is the result of an international collaborative study initiated by the National Institute for Biological Standards and Control (NIBSC) in collaboration with the European Pharmacopoeia[2]. The study was initiated in 1992. The results showed that the *in vivo* bioassay could be removed from routine batch controls without reducing safety, and were presented during an international workshop on somatropin[3].

The World Health Organization (WHO) International Standard for somatropin was used as the reference preparation.

The current international situation on somatropin was discussed in an international meeting in Strasbourg and the establishment of a conversion factor was unanimously approved[4]. A specific activity of 3.0 IU per mg of somatropin was recently adopted by the WHO group of experts (ECBS) as a conversion factor[5]; this value has been introduced in the Ph. Eur. monographs on somatropin.

AIMS OF THE STUDY

This collaborative study was performed to:

- verify the suitability of the candidate preparation to serve as a Ph. Eur. Standard for somatropin, using the analytical methodology in the proposed Ph. Eur. monograph for somatropin (951);
- determine the purity of the candidate preparation using the methodology in the Ph. Eur. monograph, and to assign values for somatropin-related impurities (desamido-somatropin, oxidised-somatropin);
- calibrate the candidate preparation in terms of the proposed International Standard for somatropin, using the assay methodology in the Ph. Eur. monograph, and to assign a content in terms of mg somatropin per mg protein;
- to harmonise the analytical procedures for quality control of somatropin among manufacturers and regulatory bodies of Europe, Japan and U.S.A.

PARTICIPANTS

Fourteen laboratories from eleven countries participated in the study: eight national laboratories and six manufacturers. The list of participants is presented at the end of the report.

(1) Somatropin, Somatropin bulk solution, Somatropin injectable preparation. Ph. Eur. monographs No. 950, 951 and 952.

(2) Human Growth Hormone. *Pharmeuropa*, **3**, special issue, (1991).

(3) Report of an international workshop on assays, standardisation and labelling requirements of somatropin, *Pharmeuropa*, **6**.1, (1994).

(4) Spieser, J-M., Quantification of somatropin: from global bioactivity measurements to molecular entity determinations. *Endocrinology and Metabolism*, **2** (Suppl B), (1995).

(5) WHO Technical Report Series, 858 (1995) 19-20.

MATERIALS AND METHODS

Materials

Candidate material. The material chosen as a candidate somatropin preparation was generously supplied by Novo Nordisk. The material was prepared by recombinant DNA technology, with a specification consistent with current commercial therapeutic somatropin preparations. The bulk somatropin was formulated by the manufacturer, and supplied in rubber-stoppered vials, freeze-dried and sealed under nitrogen.

The nominal content of each vial is:

Somatropin:	2 mg,
Glycine:	6.84 mg,
$Na_2HPO_4\ 2H_2O$:	0.98 mg,
$NaH_2PO_4\ 2H_2O$:	0.88 mg,
Mannitol:	34.2 mg.

Other preparations. Two other preparations were provided: the proposed International Standard for somatropin WHO 88/624 and a somatropin sample representing the current grade available on the U.S.A. market.

The assigned content per vial of the proposed International Standard for somatropin, WHO 88/624 is:

Somatropin:	2.09 mg,
Glycine:	20 mg,
Sodium bicarbonate:	2.5 mg,
Lactose:	2 mg,
Mannitol:	2 mg.

The U.S.A. market somatropin sample was kindly supplied by Eli Lilly. This preparation was then the current Eli Lilly in house standard, and has been offered to the USP to serve as the somatropin reference material. The nominal content of the vials is 3.0 mg somatropin per vial. There are no excipients. This material will be referred to hereafter as US sample.

Methods

Study protocol methods

Each participant was provided with the Ph. Eur. monograph for somatropin for injection and was asked to perform the following tests:

1. Quantitative determination of somatropin content in mg of the proposed Ph. Eur. standard in terms of 88/624 by performing the procedure described under "Assay".
2. Ultraviolet absorbance spectroscopy at 276 nm. The participants were asked to dissolve the substance to be examined in a 0.05 M solution of ammonium bicarbonate R to give a concentration of 1.0 mg of somatropin per millilitre. The absorbance at the maximum at 276 nm is 7.2 to 8.8. If necessary, participants were asked to correct for any light scattering due to turbidity measured at 400 nm.
3. Related proteins using a liquid chromatography procedure (e.g. a reverse phase high performance liquid chromatography, RP HPLC) using a neutral pH mobile phase and n-propanol as the organic modifier.
4. Dimers and high molecular mass aggregates using a size-exclusion high performance liquid chromatography (SE HPLC).
5. Iso-electric focusing.
6. Peptide mapping using the HPLC procedure given in the Ph. Eur. monograph.

The proposed Ph. Eur. Standard, and the US sample, were to be used as the substance to be examined. Where reference solutions were specified, these were to be prepared using both the proposed WHO International Standard for somatropin, 88/624, and also any house standard routinely in use. For each test method, it was requested that at least two separate analyses be performed. A separate analysis is here defined as one using separate vials of both the material to be examined and, if possible, the reference preparations, rather than stored aliquots of the same vial.

Each participant was sent six vials of each of the proposed Ph. Eur. Standard and of WHO 88/624, and two vials of the US sample, together with appropriate instructions for reconstitution of vial contents. For all three preparations provided, the entire contents of each vial were dissolved in appropriate diluents to give the nominal concentrations required for the various test methods.The two preparations containing excipients, the proposed Ph. Eur. Standard and the WHO 88/624, should be readily soluble in any solvent, and the procedures for preparation of test solutions given in the monograph may be used. The excipient-free US sample will not dissolve readily in water but is soluble in phosphate, tris or ammonium bicarbonate buffers above or below pH 7.5. Where adjustments are necessary to the detailed procedures for preparing test solutions, these should be recorded.

Participants were asked to return raw data for all tests performed, e.g. chromatograms and peak areas, uncorrected UV absorbance profiles, together with in-house calculations of potency, protein content, purity levels and somatropin content.

Statistical methods

The statistical analysis of the results was carried out by the NIBSC. Procedures for obtaining individual assay estimates, and estimates of dimer and higher moleculer mass content, and related proteins are set out in the Ph. Eur. monograph. Where it was clear that these procedures had been followed, in-house estimates were used without recalculation. Where in-house estimates were not obtainable in this form, estimates were obtained by calculation from the raw data provided, using, for the SE HPLC and the RP HPLC, the 4 per cent and 5 per cent reference chromatograms respectively. Overall study means were calculated as the means of individual laboratory means, rather than as the means of all estimates, in order to avoid bias in favour of laboratories submitting large numbers of estimates.

RESULTS

Quantitative determination of somatropin content

Assigned content of WHO 88/624

The International Standard 88/624 was assigned a content of 2.0 mg of protein per vial[1]. Given that the content of somatropin in the International Standard is determined by the selectivity of the assay procedure employed, it is proposed therefore that for the purposes of the present study, the content of 88/624 is taken as 2.0 x percentage of monomer per cent, the percentage of monomer being derived from the test for "dimers and higher moleculer mass impurities". From *Table 4,* therefore, the content of somatropin in 88/624 becomes 2.0 x 0.961 = 1.92 mg per vial.

Relative content of proposed Ph. Eur. Standard for somatropin and US sample by reference to 88/624

Laboratory means calculated using a defined vial content of 1.92 mg somatropin per vial for 88/624 are given in *Table 1.* The overall study mean of 2.2 mg somatropin per vial for the

(1) Bristow, A.F. et al., The first international reference standard for somatropin 88/624. *WHO Technical Report Series* 1993 and 1994.

proposed Ph. Eur. Standard for somatropin was approximately 10 per cent higher than the nominal vial content. The overall study mean for the US sample of 2.955 mg somatropin per vial is within 2 per cent of the nominal vial content. Most participants in the study were able to meet the validation criteria of a relative standard deviation of less than 2.5 per cent for six consecutive runs. Where laboratories reported independent assay estimates, the agreement was usually acceptable, the only exception being the assays from one laboratory for the US sample. The study did, however, reveal a somewhat higher degree of between-laboratory variation, with relative standard deviations of the overall study means of 4.73 and 6.72 per cent for the proposed Ph. Eur. Standard for somatropin and the US sample respectively.

Table 1. — *Determination of Somatropin Content by Size Exclusion High Performance Liquid Chromatography*

Laboratory code	Ph. Eur. Standard	U.S. sample
1	2.21	3.11
2	2.21	3.03
3	2.19	3
4	2.14	2.6
5	2.30	3.11
6	2.14	3.03
7	2.44	3.16
8	2.14	2.56
10	2.07	2.79
11	2.30	3.05
12	2.21	3.11
13	2.25	2.83
14	2.05	3.07
Overall mean	2.20	2.96
Standard deviation	0.10	0.20
RSD (per cent)	4.73	6.72
	expressed in mg/vial	

Assay performance

A number of participating laboratories reported comments on the performance of the assay. Seven of the fourteen participating laboratories reported that at the prescribed load, the detector was saturated and the relationship between response and concentration was therefore non-linear. In each case, with the exception of one laboratory who did not report assay data, these participating laboratories made appropriate alterations to the assay procedure to obtain a linear response. Three laboratories reported that they considered that the mobile phase is inappropriate, and in particular, that the pH of the mobile phase increases during the assay causing a rapid degradation of column performance.

Ultra-violet absorption spectroscopy

Fourteen laboratories determined the absorbance at 276 nm, using the above-mentioned procedure. Individual measurements, expressed as $E^{1\%}$ based on the nominal vial contents are shown in *Table 2.*

Table 2.— *Determination of Protein Content by Measure of Specific Absorbance*

Laboratory code	International Standard	Ph. Eur. Standard	U.S. sample
1	8.26	8.66	8.77
2	7.89	7.95	8.66
3	8.37	8.38	8.8
4	8.15	8.39	7.4
5	8.67	8.32	8.76
6	8.03	8.17	8.58
7		8.62	8.28
8	8.32	7.80	7.95
9	7.8	7.84	8.26
10	8.29	8.85	8.45
11	9.16	8.53	
12	8.05	8.20	8.43
13	8.46	8.38	
14	8.03	8.07	8.64
Overall mean	8.27	8.30	8.39
Standard deviation	0.36	0.31	0.44
RSD (per cent)	4.34	3.77	5.28
	expressed as specific absorbance		

In order to express these data as $E^{1\%}$ based on the actual protein contents, the following corrections were made:

- For 88/624 the protein content was taken to be 2.0 mg per vial.
- For the proposed Ph. Eur. Standard for somatropin, the somatropin content was taken to be 2.2 mg per vial. Assuming an additional content of 1.84 per cent of higher molecular mass impurities not determined in the assay, the corrected protein content was therefore taken to be 2.24 mg per vial.
- For the US sample, the somatropin content was taken to be 2.955 mg per vial. Assuming an additional content of 1.65 per cent higher molecular mass impurities not determined in the assay, the corrected protein content was therefore taken to be 3.0 mg per vial.

Means of laboratory estimates and overall study means of $E^{1\%}$, based on corrected protein contents, are shown in *Table 2*. Estimates for WHO 88/624, and the proposed Ph. Eur. Standard for somatropin were almost identical (8.27 and 8.30 respectively), whilst the estimate for the US sample (8.39) was some 2 per cent higher. The relative standard deviations for each of the preparations studied ranged from 3.7 to 5.28 per cent, in each case larger than the differences in estimated $E^{1\%}$ between the preparations. It is also notable that the estimate for $E^{1\%}$ for the proposed Ph. Eur. Standard for somatropin differs by only 1.3 per cent from that obtained for WHO 88/624 in the WHO collaborative study. No laboratory reported difficulties in performing the method, although data from one laboratory where very high light scattering values were obtained suggested poor solubility of the preparations under some conditions, and were excluded from the calculations.

Related proteins

Quantitative estimates of purity

Related proteins were determined by an RP HPLC procedure. For all three preparations, estimates were rather variable. Estimates of total impurities ranged from 1.41 per cent to 8.46 per cent for WHO 88/624, with an overall mean (*Table 3*) of 5.46 per cent. For the proposed Ph. Eur. Standard for somatropin, ten out of twelve laboratories obtained estimates between 0.98 and 2.2 per cent, although two laboratories failed to detect any significant amounts of impurities. The overall mean (*Table 3*), calculated excluding data from two laboratories was 1.58 per cent related proteins. The US sample was of an intermediate level of purity between the other two preparations, and estimates were again somewhat variable. For all three preparations, the relative standard deviations were between 25 and 50 per cent, reflecting the variability of estimates.

Table 3. — *Total Amount of Related Proteins Expressed in per cent*

Laboratory code	International Standard	Ph. Eur. Standard	US sample
1		2.15	4.00
2	3.88		1.71
3	4.19	1.65	3.67
4	7.41	2.05	4.83
5	7.4	2.20	4.1
6	3.9		
8	8.18	1.21	3.88
9		1.65	3.88
11	1.76	1.42	2.95
12	1.41	0.98	4.07
13	8.46	0.87	2.95
14	8.03	1.65	
Overall mean	5.46	1.58	3.63
Standard deviation	2.73	0.47	0.93
RSD (per cent)	50.03	29.57	25.68

Assay performance

The prescribed assay validation procedure uses oxidation with 0.01 per cent hydrogen peroxide to generate a spectrum of impurities which must then be resolved. One laboratory was unable to make the procedure work at all, and two other laboratories reported difficulties in obtaining the specified column. It has also been reported that the specified retention time was difficult to achieve, and that acetonitrile gradients at acidic pH gave better results. Typical validation chromatograms obtained for WHO 88/624 and for the proposed Ph. Eur. standard, using the specified methodology, are shown in *Figure 1*. Although every laboratory was able to resolve earlier-eluting components following oxidation, the chromatograms obtained were qualitatively variable. For the procedure employed, a specific type of column (Vydac ATP 514) was specified, and it seems unlikely that this variability derives from the use within the study of different

columns. Inspection of the relative areas of the peaks assigned to monodesamido and to the monosulphoxide growth hormones would suggest that the oxidation process specified is variable. It is also clear however that different resolutions were obtained by participants performing the same method. It should be noted that participants were not provided with a reference chromatogram (see *Figure 1*) to duplicate, which will be the case when the Ph. Eur. Standard is distributed to support the Ph. Eur. monograph. The possibility of providing a single reference preparation of oxidised somatropin rather than a specified procedure could be investigated.

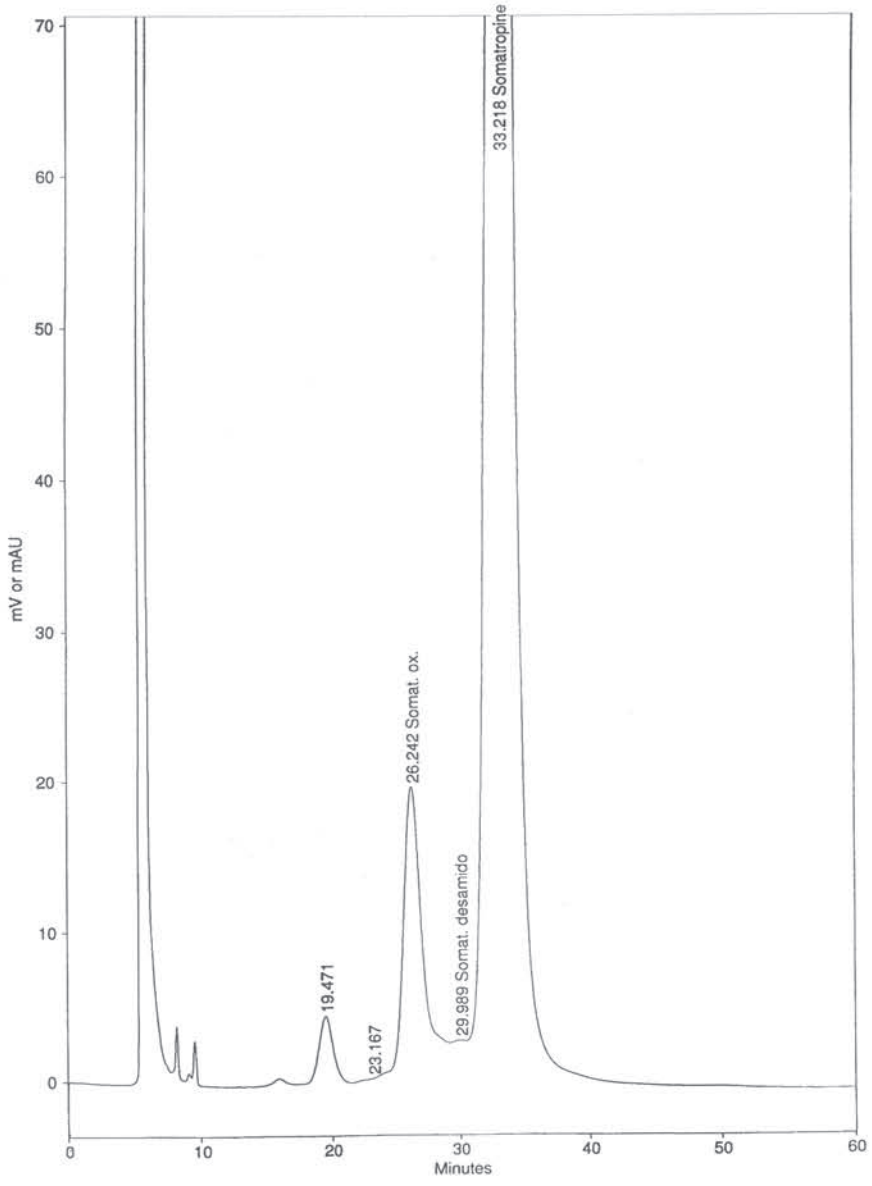

Figure 1

Dimers and higher moleculer mass impurities

Quantitative estimates of purity

Total higher molecular mass impurities were determined by SE HPLC using the conditions employed under "Assay". High molecular mass impurities were quantified by reference to the monomer peak area of a low load reference chromatogram, usually 5 per cent. Where data were

not provided in this form, estimates were obtained by re-calculation of raw data at NIBSC. Individual estimates, laboratory means of estimates of total impurities, and overall study means are shown in *Table 4*. The proposed Ph. Eur. Standard for somatropin and the US sample were of similar purity (1.78 per cent and 1.95 per cent higher moleculer mass impurities respectively), whilst WHO 88/624 was somewhat less pure (3.88 per cent).

Table 4.— *Determination of Dimers and Higher Molecular Mass Impurities*

	International Standard			Ph. Eur. Standard			U.S. Sample		
Laboratory	**D***	**HMM****	**Total**	**D***	**HMM****	**Total**	**D***	**HMM****	**Total**
1			3.50			1.30			1.05
2	0.78	2.74	3.52	0.49	0.69	1.18	0.81	0.19	1.00
3						1.93			1.54
4	3.68	1.75	5.43	0.44	0.62	1.06	0.71		
5	2.00	1.10	3.10	0.60	0.50	1.10	0.80		
6	2.04	3.16	5.20	0.80	1.56	2.36	0.80		
7						3.71			
8			4.16			1.73			2.74
9			3.14			1.20			0.86
10				0.62	0.57	1.19	0.95	1.19	2.14
11						2.77			5.49
12			3.10			0.99			0.84
13						2.70			3.03
14			3.75			1.65			0.85
Overall mean			3.88			1.78			1.95
Standard deviation			0.89			0.82			1.48
RSD (per cent)			22.86			46.35			76.02

* D = Dimers

**HMM = High Molecular Mass Impurities

Assay performance

Qualitatively the separations obtained in each laboratory for WHO 88/624 appeared very similar, and not dependent on the type of column used, although one laboratory considered that the description of the methodology should allow the use of longer or tandem columns to improve resolution. The data presented in *Table 4* suggest that the relative amounts of polymer and dimer varied between laboratories. All laboratories were able to meet the within-assay validation criterion of a relative standard deviation for 6 runs of less than 2.5 per cent. Between laboratories, however, the estimates for total high molecular mass impurities varied considerably. For the proposed Ph. Eur. Standard for somatropin estimates ranged from 0.99 per cent to 3.71 per cent, giving a relative standard deviation of 46.35 per cent, and the estimates for the US sample were even more variable. It is notable however that the variability increases as the level of impurities approaches zero, and both the proposed Ph. Eur. Standard for somatropin and the US sample complied with the Ph. Eur. monograph limit of 4 per cent in all laboratories except one laboratory for the US sample.

Isoelectric focusing

Data were reported by ten laboratories. No laboratory reported being unable to perform the method, although in some cases the load had to be reduced in order to achieve focusing. This appears to be caused by the excipients in the Standard, and is also possibly related to the instrumentation used. Few comments were received, although one laboratory reported that the method is well described and easy to perform. Another laboratory suggested that the description of the method should be altered to allow the use of immobilised pH gradients and also that consideration should be given to the use of densitometric gel scans. In every case, the method functioned acceptably, both as a qualitative test of identity, and as a semi-quantitative test for impurities.

Peptide mapping

Two laboratories performed the HPLC peptide mapping test given in the Ph. Eur. monograph. Comparisons of the maps obtained for WHO 88/624 and the proposed Ph. Eur. Standard for somatropin are shown in *Figure 2*. Within each laboratory the peaks generated were reproducible, both in retention time and peak height, and the HPLC procedure provided satisfactory resolution. However, between laboratories the peptide maps obtained for identical preparations were only approximately comparable. In view of this apparent lack of reproducibility between laboratories, the wording of the method may require revision. Nevertheless, the data from both laboratories show that the proposed Ph. Eur. Standard for somatropin is suitable to serve as a reference substance for this test of identity.

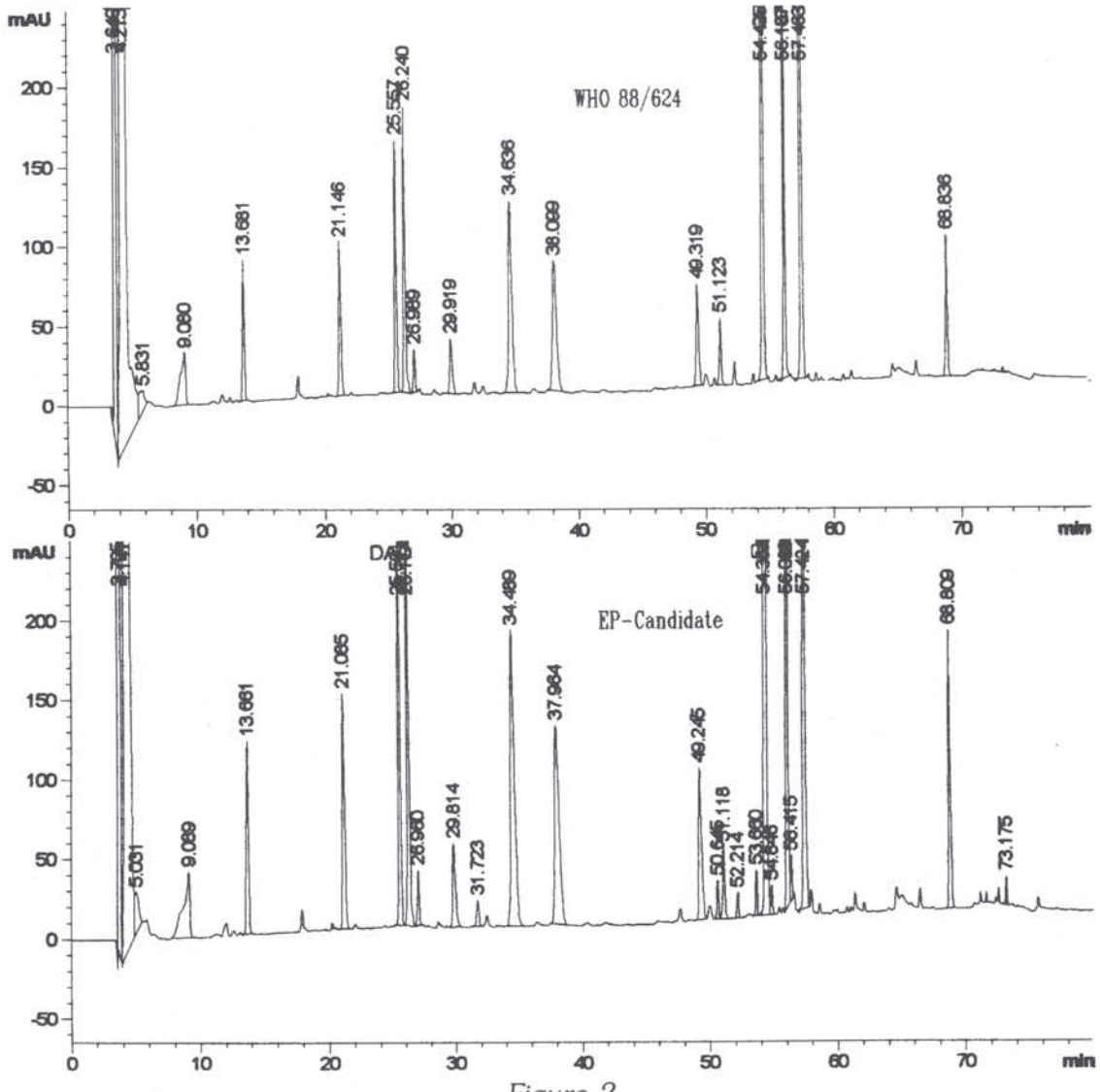

Figure 2

CONCLUSIONS

The study showed that the proposed Ph. Eur. Standard for somatropin is suitable to serve as a reference substance(*) for the current generation of analytical tests included in the Ph. Eur. monograph for somatropin, i.e. assay by SE HPLC, isoelectric focusing test, peptide mapping test and related proteins test.

Based on the results obtained it is proposed to assign to the proposed Ph. Eur. Standard for somatropin a content of 2.20 mg of somatropin monomer per vial, as determined by the under assay method (SE HPLC) in the Ph. Eur. monograph for somatropin.

The specific absorbance ($E^{1\%}$) of the proposed Ph. Eur. Standard for somatropin is 8.30.

The proposed Ph. Eur. Standard for somatropin contains 1.78 per cent dimers and higher molecular mass impurities, when determined as described in the Ph. Eur. monograph for somatropin.

The proposed Ph. Eur. Standard for somatropin contains 1.58 per cent related proteins, when determined as described in the Ph. Eur. monograph for somatropin.

Data from the collaborative study will provide a basis for refining and developing the analytical methodology as part of a continuing programme of studies on somatropin.

Data obtained for the US sample will facilitate international harmonisation.

Further improvement of the current analytical procedures applied for the quality control of somatropin have been considered in discussions involving representatives of the Ph. Eur., the Japanese Pharmacopoeia, the U.S. Pharmacopoeia, the U.S. Food and Drug Administration and manufacturers.

(*) *Somatropin CRS can be ordered from the Ph. Eur. Secretariat, Code S0947000.*

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