EXHIBIT 2

NOTICE OF
CLAIMED INVESTIGATIONAL EXEMPTION
FOR A NEW DRUG

ERYTHROPOIETIN (EPOCH)

CHUGAI PHARMACEUTICAL CO., LTD.

VOLUME 1.3

Plaintiff's Exhibit 19

CONFIDENTIAL SUBJECT TO
PROTECTIVE ORDER C.A. NO. 87-
2617-Y (D.C.MA.) C.A. NO. 87-
07200 TJH(DC.CA)CHUGAI 639

PLAINTIFF'S
EXHIBIT
19

A 119740

371

6.1.2 Biological Characterization of Protein Identity and Potency

371

6.1.2.1 Description of EPO Assays

A. Bioassays Used for EPO Project

Hormones such as EPO, have historically been difficult to assay because of the requirement for in vivo systems or in vitro systems which utilize living cells. As a consequence, a substantial body of literature has accumulated describing techniques for measuring the biological activity of EPO present in an unknown sample. The methods vary widely in terms of their accuracy and sensitivity, the amount of labor required and their in vivo relevance.

To characterize the biological properties of recombinant EPO and compare those properties with human urinary EPO, three different methods have been developed and used in this project. Each is described in detail in this section.

1. Assays of the in vitro EPO biological activity are performed by a slightly modified version of the published method of Krystal (1983). This assay measures the stimulation of proliferation within a population of isolated spleen cells enriched for erythrocyte precursors.

2. Routine assays of the in vivo activity of EPO are performed by measuring the induction of iron incorporation into erythrocytes within polycythemic mice as described by Ersley (1983).

3. The in vivo efficacy of recombinant EPO was tested in a primate species in order to characterize the in vivo activity within an animal model more relevant to human. Efficacy was measured by the ability to increase the reticulocyte fraction within the blood. Complete blood chemistry was obtained to test for unexpected reactions to the recombinant EPO. The pharmacokinetics of EPO in primates was also determined by assaying the level of EPO in the blood at various times post infusion.

A 120070

372

B. Base Standard for Establishment of Activity Unit

For assays that are designed to establish the units of biological activity within an unknown sample, it is necessary to first establish a dose/response curve for a standard of known biological activity. The unknown sample is then diluted to the extent necessary such that the response of the sample in the assay is in the linear range of the reference standard curve. Urinary EPO from Toyobo Co., Ltd. is the base standard we have used to establish unit measurement of biological activity.

The Toyobo standard consists of partially purified EPO prepared from the urine of aplastic anemia patients. It is widely used for erythropoietin research and is itself based on the internationally recognized WHO EPO standard. When new vials of standard are needed, dose response curves are obtained with the new and old standard. In every case to date these curves have demonstrated equivalent biological activity between different lots of standard (within the error range of the particular assay).

C. Measurement of EPO Protein Concentration

Accurate determination of in vitro and in vivo specific activity requires a precise measurement of protein concentration. For this purpose, samples of pure EPO with unknown protein concentration are hydrolyzed in acid and injected onto an automated Amino Acid Analyzer for quantitation of individual amino acid content using methods described in Section 6.1.1.1. The analysis also provides the amino acid composition which confirms the purity and complete hydrolysis of the sample. From the calculated recoveries and the known mole ratios of individual amino acids it is possible to extrapolate the protein concentration of the unknown sample. It should be noted that the calculated value does not consider carbohydrate content and thus measures the polypeptide concentration

375

rather than the glycoprotein concentration.

As an example of this method for measurement of EPO protein concentration, the analysis of an unknown sample is described. A homogeneous sample of EPO designated 535-87 was hydrolyzed in 6N HCL, dried and solubilized in sample buffer. Exactly 25% of the sample was injected onto the analyzer. The recovery of each amino acid was calculated by computer from the integrated area of its unique optical density peak using known standards to establish the extinction coefficient. To estimate the percent recovery, 150 pm of norleucine is included as internal control.

374

5

Figure 6.1.2.1-1

Amino Acid Composition of Reference Standard 535-87

Amino Acid	Expected Mole Ratio	Recovery 50 ul Load.nm	Observed Mole Ratio	Recovery 100 ul Load.nm	Observed Mole Ratio
Asx	12	724	11.6	1518	11.8
Thr	10	603	9.6	1258	9.8
Ser	11	488	7.8	993	7.7
Glx	19	1187	19.0	2440	19.0
Pro	8	530	8.5	1087	8.5
Gly	9	615	9.8	1271	9.9
Ala	19	1187	19.0	2478	19.3
Cys	4	-	-	-	-
Val	11	666	10.7	1406	11.0
Met	1	-	-	60	0.5
Ile	5	305	4.9	643	5.0
Leu	23	1511	24.2	3150	24.5
Norleu	145	-	-	157	-
Tyr	4	239	3.8	505	3.9
Phe	4	234	3.8	475	3.7
His	2	123	2.0	265	2.1
Lys	8	503	8.0	1075	8.4
Trp	3	-	-	-	-
Arg	13	745	11.9	1570	12.2

A 120073

Calculation of protein concentration:

In the example shown in Figure 6.1.2.1-1, 50 ul and 100 ul aliquots of an unknown sample of purified EPO were hydrolyzed and 25% of the recovered hydrolyzate was analyzed using a Beckman 6300 Amino Acid Analyzer. The high correlation between the expected and observed molar ratios indicates complete hydrolysis and confirms the high degree of purity of the sample. The quantitative recovery of the norleucine indicates 100% amino acid recovery on HPLC.

For protein concentration calculation, it is noted that 2440 pm of Glx was recovered from 25% of the 100 ul hydrolyzate indicating 9760 pm Glx/100 ul or 97.6 nmoles Glx/ml of unknown sample. Since there are 19 Glx/mole EPO, this indicates 5.1 nmoles EPO/ml. Since there are 18.4 ug protein/nmole EPO, this indicates that the unknown sample contains 94 ug EPO protein/ml.

6.1.2.2. Measurement of EPO In Vitro Biological Activity

A. Mouse Spleen Cell Proliferation Assay

For measurement of the *in vitro* biological activity of EPO, we have slightly modified an effective protocol described by Krystal (1983). Phenylhydrazine is given by injection to mice creating a drug induced anemia that results in the accumulation of erythroid precursor cells in the spleen. The spleen is removed and the population of cells, enriched for erythroid progenitors, is isolated and cultured. These cells are particularly responsive to proliferation upon addition of EPO. Thus ^{3}H-thymidine incorporation into these cells is increased well above background by culturing in the presence of EPO. Utilizing an EPO standard, a linear dose/response range is established. Unknown samples can be accurately assayed for EPO when they are diluted such that the ^{3}H incorporation is within the linear response range of the standard curve.

A 120074

7

The standard operating procedure for in vitro EPO bioassay is included in SOP #QCA014, included in Appendix A.

Validation of the in vitro EPO assay

a. Inter-assay Validation of In Vitro Bioassay

To ascertain the reproducibility of the in vitro EPO bioassay when performed on different days, a common sample was included on thirteen separate days of assay (four times as duplicates) as an internal control. The results of the assays for a six week period are shown below.

Assay #	Measured units/ml
1	608
2	624
3	591
4	608
5	758, 640
6	516
7	664
8	912
9	512, 812
10	795, 663
11	666
12	869
13	694, 596

average- 678 +/- 115 units/ml

coefficient of variability- 17.0%

The variability coefficient of 17% obtained in this sampling was significantly lower than is expected for bioassays of this nature and in several other studies, not otherwise relevant to this document, was generally in the range of 30-40%. Control samples were included in all bioassays and the results were acceptable if the control sample measured within the established variability range.

Intra-assay Validation of the EPO In Vitro Bioassay

Validation of the spleen cell bioassay was performed whenever a new lot of

serum, mice or EPO standard was introduced. The procedure is to assay the standard and a control EPO sample 10 times each and to establish a mean and coefficient of variability. If previously established acceptance criteria were not met, the validation was repeated once. A second failure would require effort to locate acceptable reagent lots.

Standard operating procedure for the in vitro bioassay validation is in SOP # QCB012, included in Appendix A.

In vitro specific activity of EPO production lots

The five production lots were each assayed for in vitro biological activity and the measured activities were converted to specific activity by using the protein concentration values measured by amino acid analysis (6.1.2.1.C). The results are shown in Figure 6.1.2.2-1. Although there is the typical variability expected for an assay of this type, all the production lots have calculated specific bioactivities in which the one sigma statistical ranges overlap. Thus, the data is consistent with the structural characterization results implying that the all of the production lots are equivalent.

375

9

Figure 6.1.2.2-1

In Vitro Specific Activity of EPO Production Lots

Lot #	Assay #	Measured Specific Activity (units/mg)
P005	1	159000
	1	137000
	1	163000
	2	223000
	2	204000
	2	140000
	3	214000
	3	214000
	average-	182000 +/- 36000
P006	1	224000
	1	286000
	2	140000
	2	110000
	2	149000
	3	193000
	3	198000
	average-	186000 +/- 59000
P007	1	137000
	1	190000
	1	206000
	2	500000
	2	428000
	2	305000
	3	352000
	3	238000
	average-	294000 +/- 125000
P008	1	246000
	1	223000
	1	169000
	2	358000
	2	356000
	3	466000
	3	293000
	3	253000
	average-	295000 +/- 94000
P009	1	214000
	1	215000
	1	253000
	2	220000
	2	244000
	2	191000
	3	159000
	3	141000
	average-	205000 +/- 39000
overall average-		234000 +/- 91000

A 120077

380

10

6.1.2.3 Measurement of EPO In Vivo Biological Activity

A. Polycythemic Mouse EPO Assay

To measure the in vivo biological activity of recombinant EPO preparations and to compare this activity to that of human urinary EPO, we have chosen to utilize a murine model system. Biological activity is measured as the increased incorporation of radioactive iron into red blood cells in blood. Basal levels of EPO in the animals is reduced by pretreating the animals with two daily infusions of concentrated murine red blood cells. This treatment induces polycythemia in the animals and results in the inhibition of de novo EPO synthesis and consequent reduction in red blood cell production. Thus the animals become particularly sensitive to exogenous EPO and exhibit a significant response to as little as 100 units. The in vivo polycythemic mouse assay is described in detail in Eralev, "Erythropoietin", p.1634 in Hematology (eds: Williams, Beutler, Eralev, and Lichtman; McGraw Hill, 3rd edition, 1983); a photocopy of the protocol from the book is attached.

Samples for in vivo activity measurements are sent to Dr. Jaime Caro, Cardeza Foundation for Hematologic Research, Philadelphia, PA. Dr. Caro has been performing the in vivo assay for a number of years in his laboratory and is considered to be one of the world's experts in this assay. Each sample is sent to Dr. Caro in form ready for injection into the mouse and is arbitrarily labeled (blind from Dr. Caro's perspective). Based on many previous calibrations of the assay to WHO EPO standards, Dr. Caro has established a standard curve relating iron incorporation to EPO activity. The results he reports to us are based on this standard calibration curve. We have validated that the in vivo activity units that Dr. Caro reports to us are equivalent to the units of biological activity obtained with the Toyobo standard we have chosen for internal

381

standardization of biological activity.

1. Validation of the mouse in vivo EPO assay

Validation was performed to test the accuracy and reproducibility of the in vivo EPO bioassay performed by Dr. Caro. For seven different weekly assays, dilutions of urinary EPO standard from Toyobo were included among the samples shipped to Dr. Caro for assay. He was unaware of the nature or dilution of these samples. The data from these samples are reported in Figure 6.1.2.3-1 and Figure 6.1.2.3-2.

38?

12

Figure 6.1.2.3-1

Validation of the Polycythemic Mouse Assay Results with Toyobo EPO

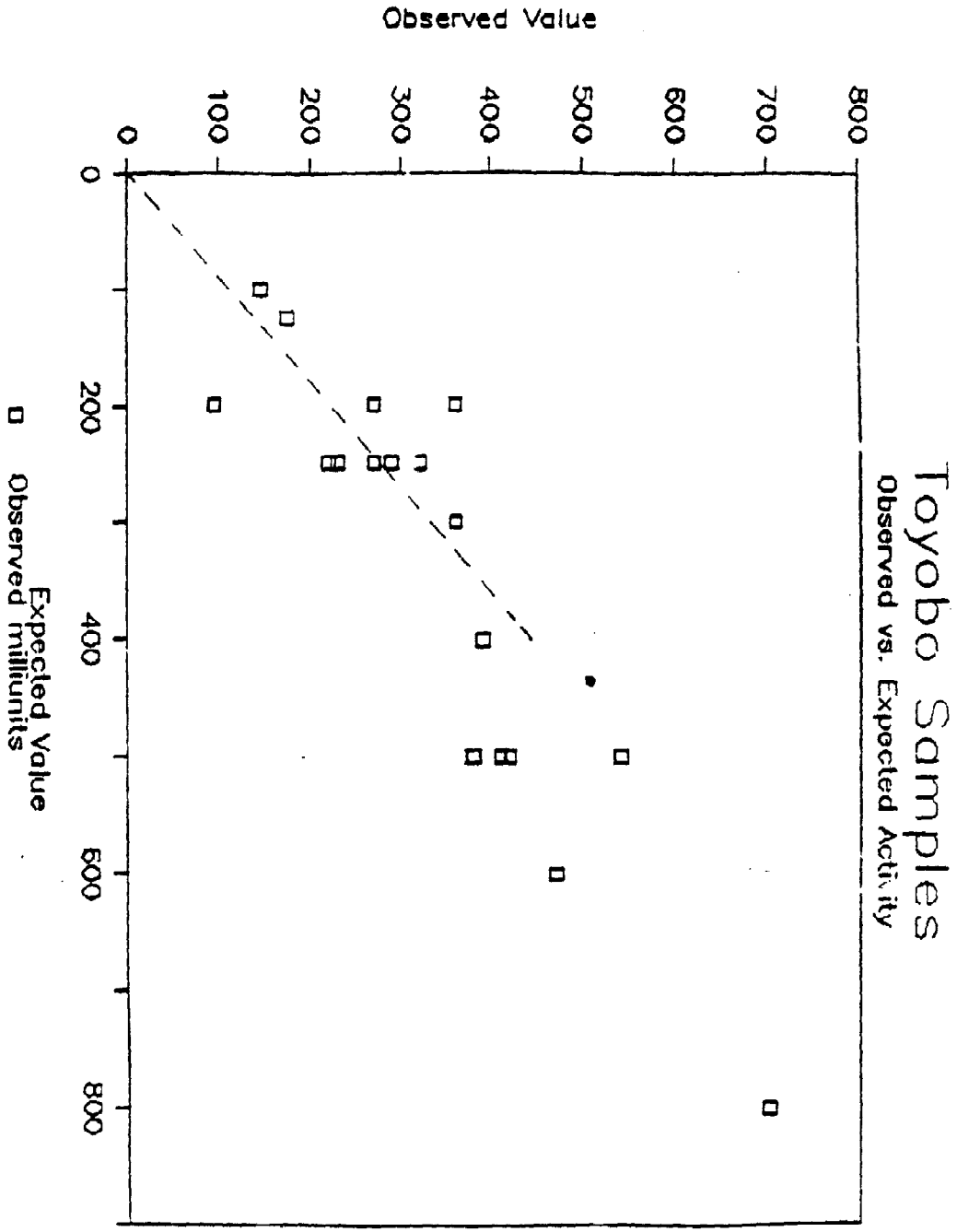
Toyobo Samples Expected		Observed	Corrected to 500
4/16	500	540	540
	250	220	440
	125	175	700
4/23	500	420	420
	250	270	540
4/30	500	540	540
	250	320	640
5/7	500	410	410
	250	290	480
	200	95	238*
5/13	500	380	380
	250	230	460
5/28	800	700	438
	600	470	392
	400	390	488
	200	270	675
6/3	300	360	600
	200	360	900*
	100	145	725

Assuming the linear range is 0-400 mUnits and eliminating two most divergent data points (*) then after adjusting all points to an arbitrary 500 mU reference point:

Average calculated in vivo activity for samples containing 500 munits/ml of Toyobo standard= 572 +/- 112 munits/ml

Figure 6.1.2.3-2 Validation of the mouse in vivo EPO assay

383



A 120081

Figure 6.1.2.3-1 shows that the expected activity (based on the st
activity on the bottle) and the observed activity (based on Dr. Caro's assay) a
within the calculated experimental error. Figure 6.1.2.3-2 indicates that th
the linear range of the in vivo assay for EPO is optimally between 100-400 mUnits
f[illegible] ml.

2. [illegible] Specific Activity of the Production Lots of EPO

Pilot plant batches P005,6,7,8, and 9 were each assayed such that at least four dilutions were within the linear range of the assay. The protein concentrations of each batch were determined by the amino acid analysis method described in Section 6.1.2.1.C. The assay results and calculated specific activity are shown in Figures 6.1.2.3-3 and 6.1.2.3-4. Estimated specific activities vary between 182,000 and 266,000 units/mg with calculated standard deviations that overlap within the one sigma statistical range (90% confidence level). The results are thus consistent with all samples having the same in vivo biological activity. Averaging the results from the five samples, the calculated specific activity of the recombinant EPO from the production lots is 231,000 +/- 51,000 units/mg with a 95% confidence level.

385

14

Figure 6.1.2.3-3

Measurement of In Vivo Specific Activity of Production Lots

Lot #	Protein Concentration mg/ml	In Vivo Activity units/ml	In Vivo Specific Activity units/mg
P005	2.0	400	200000
	2.0	390	200000
	1.5	180	120000
	1.0	210	210000
average in vivo specific activity-			182000 +/- 42000
P006	2.13	410	190000
	2.0	430	220000
	1.7	440	260000
	1.5	340	230000
	1.5	290	190000
	1.28	410	320000
	1.0	230	230000
	0.85	270	320000
average in vivo specific activity-			245000 +/- 52000
P007	2.0	325	160000
	2.0	480	240000
	1.5	320	210000
	1.0	225	220000
average in vivo specific activity-			207000 +/- 34000
P008	2.0	325	160000
	2.0	360	180000
	1.5	320	210000
	1.0	185	180000
average in vivo specific activity-			182000 +/- 21000
P009	2.0	590	300000
	1.4	390	280000
	1.4	300	210000
	0.93	220	240000
	0.7	210	300000
average in vivo specific activity-			266000 +/- 40000
average in vivo specific activity all lots-			223000 +/- 51000

A 120083

386

6.1.2.3.B Activity of Recombinant EPO in Primates

Recombinant EPO was tested for in vivo biological activity in a primate model as the most relevant animal model available. The healthy, male cynomolgous macaque (M. fascicularis) used in this study was obtained from and housed at the New England Regional Primate Center and maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the committee on care and use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. EPO expressed in DN2-3a3 cells was prepared exactly as described for the production lots. The purified protein from the final reverse-phase HPLC step was prepared for infusion by placing 4.5 milliliters of the preparation into 8 milliliters of 5% dextrose (Cutter Laboratories) which was subsequently concentrated to a final volume of 8 milliliters. Standard procedures were used to prepare and maintain samples pyrogen-free in the final formulated material. Endotoxin levels were determined by the Limilus amoebocyte lysate colorimetric assay (Whittaker Bioproducts, Walkersville, Maryland) and were undetectable (<0.1 endotoxin units ml^{-1}). The biological activity of the EPO was determined by in vitro assay to be 40,000 units/ml. EPO was infused by a continuous infusion pump (Farring Laboratories, Ridgewood, New Jersey) designed to deliver at a rate of 75-80 ul/hr. An indwelling catheter was placed in the iliac vein. EPO treatment schedule and dose were as follows:

week 1 150 units/kg/day
week 2 no EPO
week 3 300 units/kg/day
week 4 no EPO

Blood samples were taken daily after anesthetizing the animal with ketamine hydrochloride (Bristol Laboratories, Syracuse, new York) and submitted in EDTA

387

for several differential cell counts and reticulocyte counts. Body temperature and general health of the animal were also monitored on a daily basis. At weekly intervals serum was submitted for a blood chemistry profile (Vet Path, Tetenboro, New Jersey) and a serum protein analysis (Center for Blood Research, Boston, Massachusetts). As shown in Figures 6.1.2.3-5 and 6.1.2.3-6, there were no significant changes observed in the blood chemistries or serum protein analyses throughout the study period. Similarly, no temperature changes or other physical complications were observed on the administration of the protein.

388

17

Figure 6.1.2.3-5

Blood Chemistry Throughout EPO Test Period in Primate

Week #	1	2	3	4
Micro screen	.....	.....	.....	.....
Potassium	4.10	4.10	4.20	4.50
Chloride	103.00	108.00	105.00	106.00
Bun	13.00	15.00	11.00	16.00
Creatinine	1.30	1.30	1.00	1.00
Bun/Creatinine Ratio	10.00	11.50	11.00	16.00
Phosphate	4.60	3.90	3.90	4.20
Magnesium	1.64	1.65	1.69	1.52
Direct Bilirubin	0.04	0.04	0.04	0.04
Total Bilirubin	0.20	0.09	0.16	0.10
Alk. Phosphatase	44.00	34.00	26.00	30.00
G-Glutamyl Transpep.	30.00	36.00	25.00	34.00
Transaminase, SGO	40.00	19.00	40.00	17.00
Transaminase, SGP	16.00	29.00	35.00	37.00
Glucose (CS)	53.00	61.00	48.00	55.00
Sodium	142.00	146.00	146.00	142.00
Calcium	9.00	9.10	9.00	8.60
Cholesterol	77.00	84.00	89.00	91.00
Triglycerides	35.00	27.00	50.00	35.00
Total Protein	8.70	8.70	7.60	7.80
Albumin	2.50	2.90	2.70	3.30
Globulin	6.20	5.80	4.90	4.50
Alb/Glob Ratio	0.40	0.50	0.55	0.73
LDH	688.00	250.00	682.00	397.00

389

18

Figure 6.1.2.3-6

Serum Protein Analysis During ERO Test Period in Primate

week #	1	2	3	4
Total protein	8.2	8.2	7.5	7.3
Albumin	1.8	2.3	2.5	2.8
1-antitrypsin	50	52	18	7
Haptoglobin	190	127	107	94
Transferrin	235	265	255	290
Orosomucoid	32	68	48	35
	72	97	92	97
C3	140	150	162	154
IgG	3000	2480	1640	1400
IgA	140	194	195	180
IgM	24	20	30	32
Properdin Factor B	17	18	20	12
lipoprotein	10	12	22	18

Fibrinogen Test

week #	1	2
Fibrinogen	273 mg/dl	223 mg/dl

390

Figure 6.1.2.3-7 plots the reticulocyte count during the course of treatment. Also noted on the figure are the days at which blood samples were taken for analysis of the blood chemistry and serum protein. The figure clearly shows that significant increases in reticulocyte percentages were observed following infusion of the recombinant EPO and decayed to normal levels within a week following termination of EPO infusion. No other hematological parameters including white blood cell count, hematocrit, hemoglobin or platelet count changed significantly during the course of the study. The continuous infusion of 5% dextrose alone failed to demonstrate this increase in reticulocytes over the same time period.

6.1.2.4 Comparison of Biological Activity of Recombinant and Urinary EPO

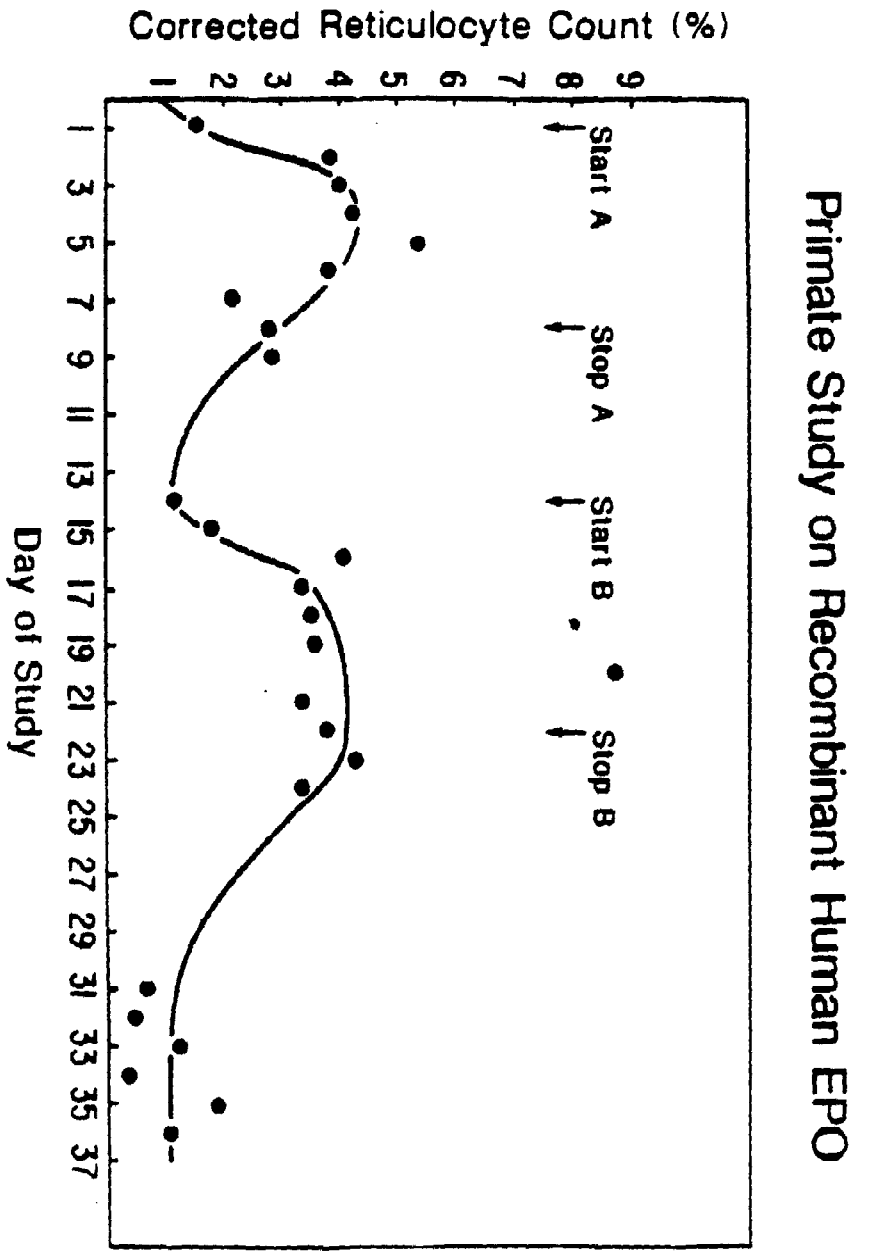
A. Purification of Human Urinary EPO

Human urinary EPO was extracted and purified from the urine of patients with aplastic anemia by the following procedure:

- Continuous centrifugation to remove precipitate
- Concentration and dialysis with hemofilter PAN-140
- DEAE-Sephacel adsorption and batchwise desorption
- Concentration and dialysis with hemofilter PAN-140
- Lyophilization
- Boil 3 minutes in buffered 2% SDS solution to destroy neuraminidase
- 50-90% ethanol fractionation
- Blue-Sepharose affinity chromatography
- Reverse phase HPLC
- Reverse phase HPLC
- TSK G3000SW GPC FPLC

391

Figure 6.1.2.2-7



A 120089

392

B. Comparison of Recombinant and Urinary EPO In Vitro Specific Activity

Purified urinary EPO was subjected to amino acid analysis as described in 6.1.2.1.C. The results of two analyses are shown in Figure 6.1.2.4-1.

393

21

Figure 6.1.2.4-1

Amino Acid Composition of Urinary Human EPO

Amino Acid	Expected Mole Ratio	Recovery Analysis #1 (nm)	Recovery Analysis #2 (nm)	Recovery Average (nm)	Observed Mole Ratio
Asx	12	1164	1167	1166	12.6
Thr	10	950	954	952	10.3
Ser	11	833	827	830	9.0
Glx	19	1752	1759	1756	19.0
Pro	8	728	736	732	7.9
Gly	9	836	836	836	9.0
Ala	19	1722	1727	1725	18.7
Cys	4	-	-	-	-
Val	11	990	995	993	10.7
Met	1	56	56	56	0.6
Ile	5	431	438	435	4.7
Leu	23	2120	2121	2120	23.0
Tyr	4	369	372	371	4.0
Phe	4	418	425	421	4.6
His	2	182	181	182	2.0
Lys	8	742	740	741	8.0
Trp	3	-	-	-	-
Arg	13	1037	1035	1036	11.2

CONFIDENTIAL SUBJECT TO PROTECTIVE ORDER C.A. NO. 87-2617-Y (D.C.MA.) C.A. NO. 87-07200 TJH(DC.CA)CHUGAI 981

A 120091

394

The amino acid composition confirms the purity of the urinary EPO and demonstrates the equivalence to recombinant EPO. The analysis also permits the precise determination of EPO protein concentration.

The urinary EPO was assayed for its in vitro activity by the spleen cell proliferation method. Four dilutions of urinary EPO were assayed, all within the linear range of the assay. The results are shown in Figure 6.1.2.4-2. The in vitro specific activity of urinary EPO is very close to that calculated for recombinant EPO (6.1.2.2.A.3) and well within the statistical range of uncertainty for these assays.

395

Figure 6.1.2.4-2

In Vitro Specific Activity of Human Urinary EPO

Protein Concentration ng/ml	In Vitro Activity mu/ml	In Vitro Specific Activity units/mg
3.6	700	190000
1.8	389	220000
0.9	215	240000
0.45	99	220000

Average in vitro specific activity= 218000 +/- 21000 units/mg

396

C. Comparison of Recombinant and Urinary EPO In Vivo Specific Activity

Highly purified human urinary EPO with precisely measured protein concentration (Section 6.1.2.4.B) was assayed for in vivo EPO activity by the polycythemic mouse method. Four dilutions within the 100-1000 munits/ml range were assayed and the results reported in Figure 6.1.2.4-3. The in vivo specific activity of the highly purified urinary EPO appears to be approximately 65% that of recombinant EPO while the in vitro specific activity (6.1.2.4.B) of urinary EPO appears identical to recombinant EPO. This implies that the urinary protein is capable of eliciting the same biological effect on responsive cells but it may be inactivated or cleared more rapidly than recombinant EPO when injected into a living animal. It is well known that the in vivo biological activity of glycoproteins is affected by the extent to which the carbohydrate chains are capped with sialic acid. Proteins containing uncapped chains are much more rapidly cleared from the bloodstream than fully sialated glycoproteins and therefore have reduced activity. Since urinary EPO is purified from much cruder starting material than recombinant EPO, it is probable that urinary EPO is more exposed to the neuraminidase enzymes which can desialate glycoproteins. This may well explain the reduced in vivo activity of the urinary EPO.

397

25

Figure 6.1.2.4-3

In Vivo Specific Activity of Human Urinary EPO

Protein Concentration ng/ml	In Vivo Activity munits/ml	In Vivo Specific Activity units/mg
3.6	330	92000
1.8	260	140000
0.9	155	170000
0.45	75	170000

average in vivo specific activity- 143000 +/- 37000 units/mg