

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : C12Q 1/00</p>	<p>A1</p>	<p>(11) International Publication Number: WO 91/15594 (43) International Publication Date: 17 October 1991 (17.10.91)</p>
<p>(21) International Application Number: PCT/US91/02121 (22) International Filing Date: 29 March 1991 (29.03.91) (30) Priority data: 505,307 5 April 1990 (05.04.90) US (71) Applicant: HYGEIA SCIENCES, INC. [US/US]; 330 Nevada Street, Newton, MA 02160-1432 (US). (72) Inventors: CLOUGH, Kathleen, M. ; 36 Spruce Street, Acton, MA 01720 (US). COLE, Francis, X. ; 75 Kirkland Drive, Stow, MA 01775 (US). (74) Agent: MARSH, James, H., Jr.; Staas & Halsey, 1825 K Street, N.W., Washington, DC 20006 (US).</p>		<p>(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i></p>
<p>(54) Title: TOTAL GONADOTROPAL ALPHA PEPTIDE CHAIN ASSAY</p>		
<p>(57) Abstract An assay procedure for predicting the onset of the ovulation in a human subject. Urine samples are obtained on consecutive days and assayed for total gonadotropal alpha peptide chain content. The alpha chain content surges to indicate ovulation and the onset of the fertile period.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

- 1 -

TOTAL GONADOTROPAL ALPHA PEPTIDE CHAIN ASSAY

BACKGROUND OF THE INVENTIONField of the Invention

The present invention relates to assays for determining
5 and/or detecting hormones in mammalian body fluids and in
particular to assays for determining and/or detecting the
luteinizing hormone surge which results in rupture of the
preovulatory follicle and release of the ovum at ovulation.
More particularly, the invention relates to the use of total
10 gonadotropal alpha peptide chain content in urine as an
enhanced indicator of luteinizing hormone content. Even more
particularly, the invention relates to an immunoassay suitable
for testing for constituents in human urine to determine the
human fertile period, that is, the period in which viable
15 sperm and a viable ovum may be present simultaneously in the
female reproductive tract.

The Prior Art Background

For a number of reasons it may be clinically and/or
diagnostically desirable to determine the presence and/or
20 concentration of constituents in mammalian body fluids such
as blood serum or urine. In some instances, mammalian
hormonal activity and/or metabolism create situations where
surges in concentrations of hormones or metabolites in body

- 2 -

fluids may be chronologically related to other events. In particular, a surge in the concentration of luteinizing hormones (LH) in human urine may be used to ascertain the fertile period of the menstrual cycle. For couples experiencing difficulty in conceiving there is a need for identifying the optimum period for intercourse or artificial insemination.

The human menstrual cycle is governed by the cyclical release of hormones from the female glands and organs. Such release is predictable and specifically related to ovulation by which ova are released from the ovaries and the lining of the uterus is made ready for pregnancy. Eventually, the released hormones and/or metabolites thereof find their way into the urine. The specific biological phenomena are described in detail in a published dissertation of Kevin J. Catt and John G. Pierce entitled "Gonadotropic Hormones of the Adenohypophysis", which appears as Chapter 3 (pp 75-114) of a treatise of Yen, S.S.C. and Jaffee, R.B., REPRODUCTIVE ENDOCRINOLOGY, 2d ed., W.B. Saunders, Philadelphia (1978). And suffice it to say, that during a normal menstrual cycle, the level of LH in female serum surges to cause the preovulatory follicle to rupture and release the ovum. This process is known as ovulation. The LH surge may be detected in female urine approximately 8 to 24 hours after the surge occurs in the blood. The surge of LH in human urine has thus been used as an indication that the fertile period is ongoing

- 3 -

or about to occur and a number of commercial assays for detecting the fertile period have been based on the measurement of LH concentrations in human urine.

5 Gonadotropins such as LH, follicle-stimulating hormone (FSH), human chorionic gonadotropin (hCG) and thyroid-stimulating hormone (TSH) are all well known hormones as discussed in the Catt et al. dissertation identified above. These hormones are all formed from two peptide chains (an alpha chain and a beta chain) which are non-covalently joined
10 to present the intact hormone. The chains are capable of existing separately; however, they must be joined together to create a biologically active hormone. The alpha chains of LH, FSH, hCG and TSH are essentially identical but the beta chains are all different.

15 As is known in the art, the intact (holo) hormones and the free alpha and beta chains may be distinguished and assayed separately using various combinations of antibodies to private, public and/or conformational epitopes. In this regard, a public epitope is one that is accessible without
20 regard to whether the chain is free or combined, a private epitope is one that is accessible only on free chains and a conformational epitope is one that is not available on either chain alone but only on an intact hormone. And, as indicated above, commercial fertile period assays have previously been
25 configured to determine the LH surge by assaying for the intact hormone.

- 4 -

It has also been recognized that LH assays could potentially be directed to either total or free LH beta chain. However, there has been no suggestion in the prior art that there might be a sufficient correlation between preovulatory total alpha chain content and intact LH surges in urine to base a fertile period assay on total alpha chain. Even more so there has been no suggestion in the prior art that total alpha chain not only surges contemporaneously with LH but surges to a much greater level so that the assay is much more sensitive and precise.

SUMMARY OF THE INVENTION

In accordance with the invention it has been discovered that in the urine of normal cycling women the total content of gonadotropical alpha peptide chains in urine is considerably greater than the total LH beta chain or intact LH hormone content. It has also been discovered that the levels of alpha chain, beta chain and holohormone all increase in a coordinated fashion at mid cycle. Thus, it has been observed, in accordance with the invention, that the preovulatory surge of the holohormone is accompanied by a simultaneous magnified surge in total alpha chain. This facilitates construction of an assay having an enhanced signal to noise ratio compared to other assays based on the holohormone or on free or total beta chain. Measurements have shown that the molar equivalent concentration of gonadotropical alpha chain at the mid cycle

- 5 -

surge is approximately five to ten times greater than the molar equivalent concentrations of the holohormone or the beta chain.

To take advantage of the foregoing observed phenomena, the invention provides an assay procedure which includes the steps of obtaining a sample of body fluid from a mammal and analyzing said sample to determine or detect gonadotropal alpha peptide chains without regard to whether the latter are free or part of an intact hormone. In another aspect of the invention, an assay procedure is provided for determining preovulatory surging of luteinizing hormone which includes the steps of obtaining a plurality of samples of body fluid from a host and comparing the total gonadotropal alpha peptide chain content of said samples. In yet a further more specific aspect of the invention, an assay procedure is provided for predicting the onset of ovulation in a human subject which comprises obtaining a urine sample from a host on each of a plurality of consecutive time periods, analyzing each of said samples to determine total gonadotropal alpha peptide chain content thereof, and observing a surge in said total gonadotropal alpha peptide chain content as a prelude to ovulation.

In the more specific aspects of the invention an assay procedure is provided wherein the samples are analyzed using an immunological procedure. The immunological procedure may include the step of forming a sandwich using two different

- 6 -

antibodies, each of said antibodies being specifically bindable to a respective different binding site on the alpha chain.

In a preferred form of the invention, the assay procedure
5 may include a sandwich ELISA procedure. And in a particularly preferred form of the invention, the samples are obtained on consecutive days and comprise first morning urine samples.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a chart which compares the mid cycle
10 preovulatory surges of intact LH and total alpha chain on consecutive days of 7 cycles;

Figure 2 is a standard curve relating total alpha chain content to spectrophotometric absorbance values; and

Figure 3 illustrates the total alpha content of urine
15 during essentially an entire menstrual cycle.

DETAILED DESCRIPTION OF THE INVENTION

As set forth above, the present invention is based on the discovery of the occurrence of two unexpected phenomena during the menstrual cycle of normally cycling women. First it was
20 discovered that the traditional mid cycle surge of intact LH in the system that triggers ovulation is accompanied by a contemporaneous surge in the total level of gonadotropeal alpha peptide chains in the system. Additionally it has been discovered that the alpha chain surge is greatly magnified
25 relative to the surge of the holohormone.

- 7 -

These phenomena were discovered by conducting dot blot analyses to determine the epitope specificity of certain LH antibodies. The antibodies investigated were HK1 2G9, LH25 2B10, INN-132, INN-72, LH26-2G9, WSP-2G5 and WSP-2B6. The INN antibodies are commercially available from I.D.C. (A-6080
5 Innsbruck/IGLS, Gsturnsteig 10, AUSTRIA) and the WSP antibodies are available commercially from Western States Plasma (3887 Alta Vista Drive, Fallbrook, CA). The other antibodies are available in-house at Hygeia Sciences, Inc.

10 LH antigens (free beta chain, free alpha chain and intact holohormone; obtained from Scripps) were bound to nitrocellulose at a concentration of 5 $\mu\text{g/ml}$ in 0.2 M carbonate buffer (pH 8.0) via a 96-well device (Bio-Rad, Richmond, CA) by gravity filtration. The nitrocellulose was
15 then exposed to a solution of 2% BSA in 0.2 M Carbonate, pH 9.0 to block remaining binding sites. The monoclonal LH antibodies at a concentration of 25 $\mu\text{g/ml}$, 100 $\mu\text{L/well}$ were gravity filtered through a membrane. Unbound antibody was removed by washing the wells three times with 300 μL of 0.2 M
20 Carbonate with 0.1% Tween 20 using vacuum filtration. Anti-mouse IgG labelled with Alkaline Phosphatase (Sigma Chemical, St. Louis, MO) diluted 1:1200 in blocking buffer, 100 $\mu\text{L/well}$, was gravity filtered, followed by three washes using the above mentioned wash buffer and vacuum filtration. A final wash
25 with distilled water was used to remove any residual tween.

- 8 -

The membrane was removed from the device and exposed to substrate consisting of .01% Nitro Blue Tetrazolium, .004 M CaCl₂, and 0.5 mg/mL Indoxyl Phosphate in Veronal-Acetate Buffer, pH 9.6.

5 The results obtained were straight forward with very little background and it was determined that HK1 2G9 antibody is specific for a public epitope on the LH beta chain, LH25 2B10 antibody is specific for a public epitope on the LH beta chain, INN-132 antibody is specific for a public epitope on
10 the alpha chain, INN-72 antibody is specific for a private epitope on the alpha chain, LH26-2G9 antibody is specific for a private epitope on the beta chain, WSP-2G5 antibody is specific for a public epitope on the alpha chain, and WSP-2B6 antibody is specific for a conformational epitope on the
15 holohormone.

Pairs of these antibodies were thus used to develop sandwich ELISA assays for specific analytes. For example, WSP-2B6 (holohormone) and WSP-2G5 (alpha) could be used to assay for the intact hormone; INN-132 (alpha) and WSP-2G5
20 (alpha) could be used to assay for total alpha chain; and LH25 2B10 (beta) and HK1 2G9 (beta) could be used to assay for total beta chain.

Immunoassays were conducted using the foregoing pairs of antibodies and a 96 well Immulon plate. WSP-2B6, INN-132 and
25 LH25 2B10 antibodies were used as the respective capture antibodies and the other antibody of each pair was labelled

- 9 -

with horse radish peroxidase (HRP). The capture antibodies were dispersed in 0.05 M PBS (pH 7.35) at different concentrations. The concentration of the WSP 2B6 antibody was 10 $\mu\text{g}/\text{ml}$; the concentration of the INN-132 antibody was 5 $\mu\text{g}/\text{ml}$; and the concentration of the LH25 2B10 antibody was 1 $\mu\text{g}/\text{ml}$. Each of these dispersions was coated (100 μl) in a plurality of wells and incubated overnight at room temperature.

For blocking, the plates were decanted and the wells were filled with 0.1 M Tris pH 8.0 containing 2% BSA and 20% Sucrose and incubated for at least 30 minutes. Prior to use the blocking solution was decanted and the plate was rinsed with tap water. For long term storage, the blocking solution could be decanted and the plate tapped several times onto paper towels to remove residual liquid and then placed in a vacuum desiccator.

The HRP labelled WSP-2G5 antibody was dispersed in 0.1 M Tris (pH 8.0) containing 1% bovine serum albumin (BSA), 1% polyethylene glycol (PEG-8000), and 0.1% Tween 20 at a concentration of 1 $\mu\text{g}/\text{ml}$, and the HRP labelled HK1 2G9 was dispersed at a concentration of 0.5 $\mu\text{g}/\text{ml}$. Urine samples were diluted 1:2 or greater in 0.1 M TRIS, 1% BSA.

The assays were conducted using 50 μl of diluted sample and 50 μl of HRP labelled antibody dispersion. Incubation was allowed to proceed for one hour at room temperature and then the plates were decanted and washed 6 to 8 times. Color was

- 10 -

developed using 100 μ l of TMB substrate solution. The intensity of the color was detected on a spectrophotometric plate at absorbance 630, and results were evaluated using standard curves similar to the one presented in Fig. 2. The results for seven different panelists are set forth in Table I below and are summarized in Fig. 1. The data of Table I is set forth in terms of pica molar concentrations. From Table I and Fig. 1 it can be seen that total alpha chain content surges contemporaneously with the surge of intact LH and that the total alpha chain surge is much greater than the surges of other components.

TABLE I
SURGE COMPARISON

Panelist #446					
	Cycle Day	LH Intact	Total Beta	Total Alpha	Free Alpha
15	12	0	0	106	0
	13	0	0	114	0
	14	75	121	1995	702
20	15	0	0	387	99
	16	0	41	212	-
Panelist #448					
	Cycle Day	LH Intact	Total Beta	Total Alpha	Free Alpha
25	9	0	0	88	-
	10	0	37	242	46
	11	138	112	1510	314
	12	41	142	397	99
	13	0	91	150	36

Panelist #449

	<u>Cycle Day</u>	<u>LH Intact</u>	<u>Total Beta</u>	<u>Total Alpha</u>	<u>Free Alpha</u>
5	10	0	0	176	-
	14	0	0	140	-
	15	0	0	254	-
	16	0	45	350	107
	17	55	49	398	127
	18	184	169	1135	250

Panelist #450

	<u>Cycle Day</u>	<u>LH Intact</u>	<u>Total Beta</u>	<u>Total Alpha</u>	<u>Free Alpha</u>
15	10	0	34	326	119
	11	0	53	220	101
	12	300	253	1970	667
	13	146	161	1400	-
	14	76	331	496	371

Panelist #451

	<u>Cycle Day</u>	<u>LH Intact</u>	<u>Total Beta</u>	<u>Total Alpha</u>	<u>Free Alpha</u>
25	15	-	-	119	0
	16	0	0	219	32
	17	162	116	1055	198
	18	0	49	203	42
	19	0	236	290	57

Panelist #453

	<u>Cycle Day</u>	<u>LH Intact</u>	<u>Total Beta</u>	<u>Total Alpha</u>	<u>Free Alpha</u>
30	10	0	0	179	-
	11	0	0	312	64
	13	68	95	2010	496
	14	0	0	364	33
	15	0	199	535	-

- 12 -

Panelist #454

	<u>Cycle</u> <u>Day</u>	<u>LH</u> <u>Intact</u>	<u>Total</u> <u>Beta</u>	<u>Total</u> <u>Alpha</u>	<u>Free</u> <u>Alpha</u>
	14	0	55	347	-
5	15	0	0	149	54
	16	207	172	1185	209
	17	230	284	1062	159
	18	88	376	468	111

The foregoing results are convincing and strongly prove
 10 that a fertile period assay may be based on an assay for total
 alpha chain. Such an assay is described in Example I
 hereinbelow.

EXAMPLE I

Total Alpha Chain Assay

15 Buffers

1. 0.1 M Tris, pH 8.0 with 1% Bovine Serum Albumin (BSA). This buffer is used to prepare alpha chain standards and as a diluent for the urine sample.
2. 0.1 M Tris, pH 8.0 with 1% BSA, 1% Polyethylene Glycol (PEG) of molecular weight 8000 and 0.1% Tween
 20 20. This buffer is used as a diluent for the conjugates.

Antibodies

1. Anti-Alpha, designated INN-132. This antibody is
 25 used as the capture antibody.
2. Anti-Alpha, designated WSP-2G5. This antibody is against a different epitopic site than the first and will make a sandwich in the presence of alpha chain

- 13 -

consisting of the first antibody, the alpha chain and the second antibody. This system will measure both the free alpha chain and the alpha chain which is bound to beta chain. This second anti-alpha antibody is labelled with an enzyme, Horseradish Peroxidase (HRP) so that detection can be accomplished visually with the addition of a substrate or chromogen that will change color in the presence of HRP.

5

10 Plates

Immulon II 96 well polystyrene plates (Dynatech) are used for coating the capture antibody. The capture antibody is diluted in 0.05 M Phosphate Buffered Saline (PBS) to a concentration of 10 $\mu\text{g/mL}$. 100 μL is added per well and incubated overnight. The wells are decanted and then filled with a solution of 0.1 M Tris, pH 8.0 containing 2% BSA and 20% sucrose to block any remaining binding sites on the plastic wells. The blocking solution is incubated for a minimum of 30 minutes after which time the plate is decanted and rinsed with tap water. The plate is now ready for use.

15

20

Standards

For the total alpha assay, either purified alpha chain or purified intact LH can be used. Both are purchased from Scripps Laboratories, San Diego, CA. The concentrations used

- 14 -

are expressed in Molarity and range from 250 pM to 15.5 pM. Using these standards in an assay allows us to develop a standard curve from which unknown samples can be interpolated.

Tetramethylbenzidine (TMB) Substrate

5 TMB substrate is prepared using two solutions. The first, TMB solution, is prepared by adding 4.75 g of tetramethylbenzidine to 3.8 L of methanol. This solution should be protected from light. The second solution is substrate buffer with stannate and is prepared by admixing
10 5.35 g citric acid, 75.27 g sodium phosphate dibasic, 0.31 g sodium stannate, 5.2 mL 30% hydrogen peroxide and 0.26 g thimerosal in sufficient purified water to bring the total volume to 5.2 L. Final pH should be 4.9 to 5.1. This solution should never come in contact with metal. For use,
15 3 parts of the TMB solution is mixed with 7 parts of the substrate buffer.

Assay Format

50 μ L of standard or diluted unknown urine is added in duplicate to wells. 50 μ L of diluted anti-alpha conjugated to
20 HRP is added to each test well. Incubation is for one hour at room temperature. The wells are then decanted and washed with tap water to remove any unbound labelled antibody. 100
 μ L of the chromogen substrate is then added. In the presence of HRP the clear substrate solution will change to a blue
25 color. The amount of bound HRP labelled antibody is proportional to the concentration of alpha chain present in

either the standard or the unknown sample and therefore the color generated is in direct proportion to the alpha concentration. The intensity of the color solution is detected by a spectrophotometric plate reader at absorbance 5 630. A standard curve is drawn (Fig. 2) based on the average of the absorbance values (Table II) and the total alpha chain content of unknown samples is determined by interpolation from the standard curve and the pM multiplied by the dilution factor (Table III).

10

TABLE II
TOTAL ALPHA ASSAY

STANDARDS

	<u>[LH]</u> <u>pM</u>	<u>Absorbance</u> <u>630</u>	<u>Average</u> <u>Absorbance</u> <u>Value</u>
15	0.0	0.037	
		.015	.026
	15.5	.071	
		.056	.064
20	31.0	.110	
		.108	.109
	62.5	.206	
		.206	.206
	125.0	.407	
		.448	.427
25	188.0	.694	
		.619	.656
	250.0	.964	
		.952	.958

- 16 -

TABLE III

UNKNOWN

	<u>Cycle Day</u>	<u>Dilution Factor</u>	<u>Absorbance 630</u>	<u>Average Absorbance Value</u>	<u>PM</u>
5	2	2	.113	.101	61
			.107		
	3	2	.431	.472	264
			.451		
10	4	2	.280	.216	149
			.240		
	5	2	.201	.209	127
			.218		
15	6	2	.164	.176	106
			.188		
	7	2	.153	.144	85
			.136		
	8	2	.389	.364	214
			.340		
20	9	2	.147	.148	88
			.149		
	10	2	.465	.413	242
			.362		
25	11	2	1.780	1.849	> linear
			1.918		
	11	8	.669	.648	1490
			.627		
	11	16	.315	.323	1530
			.332		
30	12	2	.666	.698	397
			.730		
	13	2	.253	.264	150
			.275		
	14	2	.273	.308	183
			.343		
35	15	2	.166	.168	101
			.170		
	16	2	.179	.178	108
			.178		
40	17	2	.191	.204	124
			.218		
	18	2	.107	.110	63
			.114		
45	19	2	.100	.100	43
			.100		

- 17 -

	<u>Cycle</u> <u>Day</u>	<u>Dilution</u> <u>Factor</u>	<u>Absorbance</u> <u>630</u>	<u>Average</u> <u>Absorbance</u> <u>Value</u>	<u>PM</u>
5	20	2	.184 .161	.172	104
	21	2	.184 .162	.173	104
	22	2	.117 .102	.109	62
10	23	2	.139 .144	.141	83
	24	2	.342 .390	.366	215

The foregoing data is plotted in graphical form in Fig. 3 where it can be seen that the total alpha chain surge indicating the fertile period at day 11 is substantial.

- 18 -

WE CLAIM:

1. In an assay procedure, the steps of obtaining a sample of body fluid from a mammal and analyzing said sample to determine or detect gonadotrope alpha peptide chains without regard to whether the latter are free or part of an intact hormone.
2. In an assay procedure for determining preovulatory surging of luteinizing hormone, the steps of obtaining a plurality of samples of body fluid from a host and comparing the total gonadotrope alpha peptide chain content in said samples.
3. An assay procedure for predicting the onset of ovulation in a human subject comprising obtaining a urine sample from a host on each of a plurality of consecutive time periods, analyzing each of said samples to determine total gonadotrope alpha peptide chain content thereof and observing a surge in said total gonadotrope alpha peptide chain content as a prelude to ovulation.
4. An assay procedure as set forth in claim 3, wherein the samples are analyzed using an immunological procedure.

5. An assay procedure as set forth in claim 4, wherein said immunological procedure includes the step of forming a sandwich using two different antibodies, each of said antibodies being specifically bindable to a respective different binding site on said alpha chain.

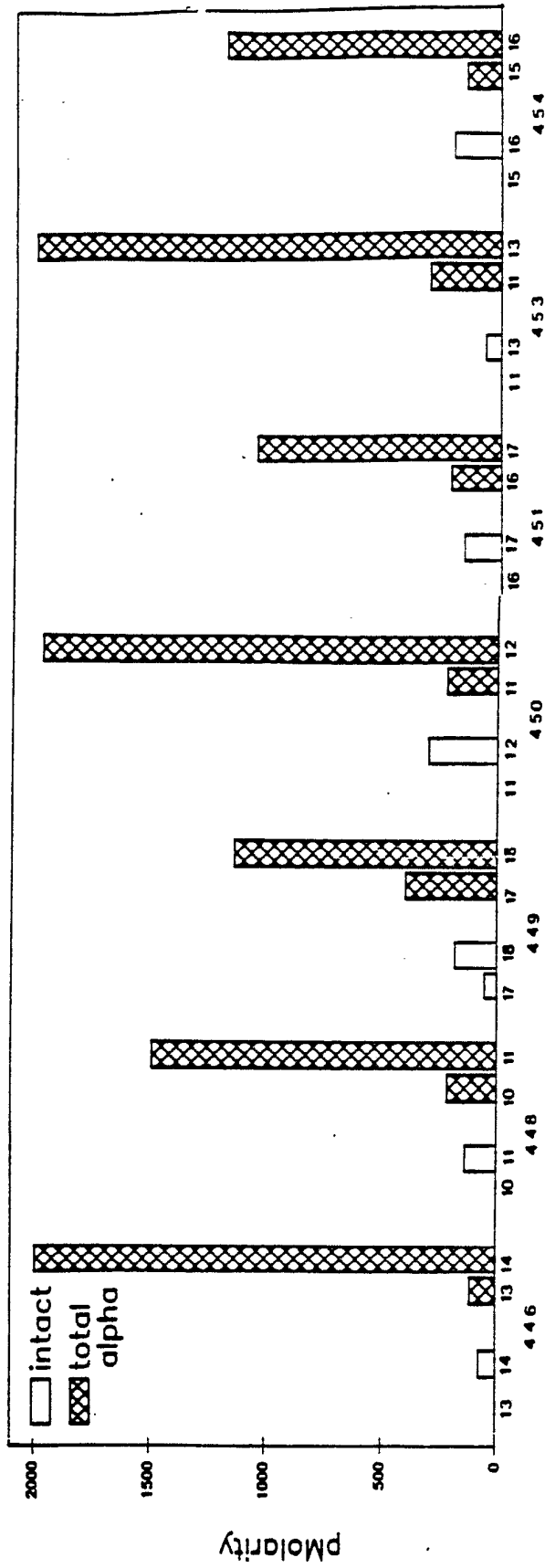
6. An assay procedure as set forth in claim 5, wherein said immunological procedure comprises a sandwich ELISA procedure.

7. An assay procedure as set forth in claim 3, wherein said consecutive time periods are days and said urine samples are first morning urine samples.

8. An assay procedure as set forth in claim 6, wherein said consecutive time periods are days and said urine samples are first morning urine samples.

1/3

Surge Comparison Trials



Two consecutive days of 7 cycles.

FIG. 1

2/3

TOTAL α STANDARD CURVE

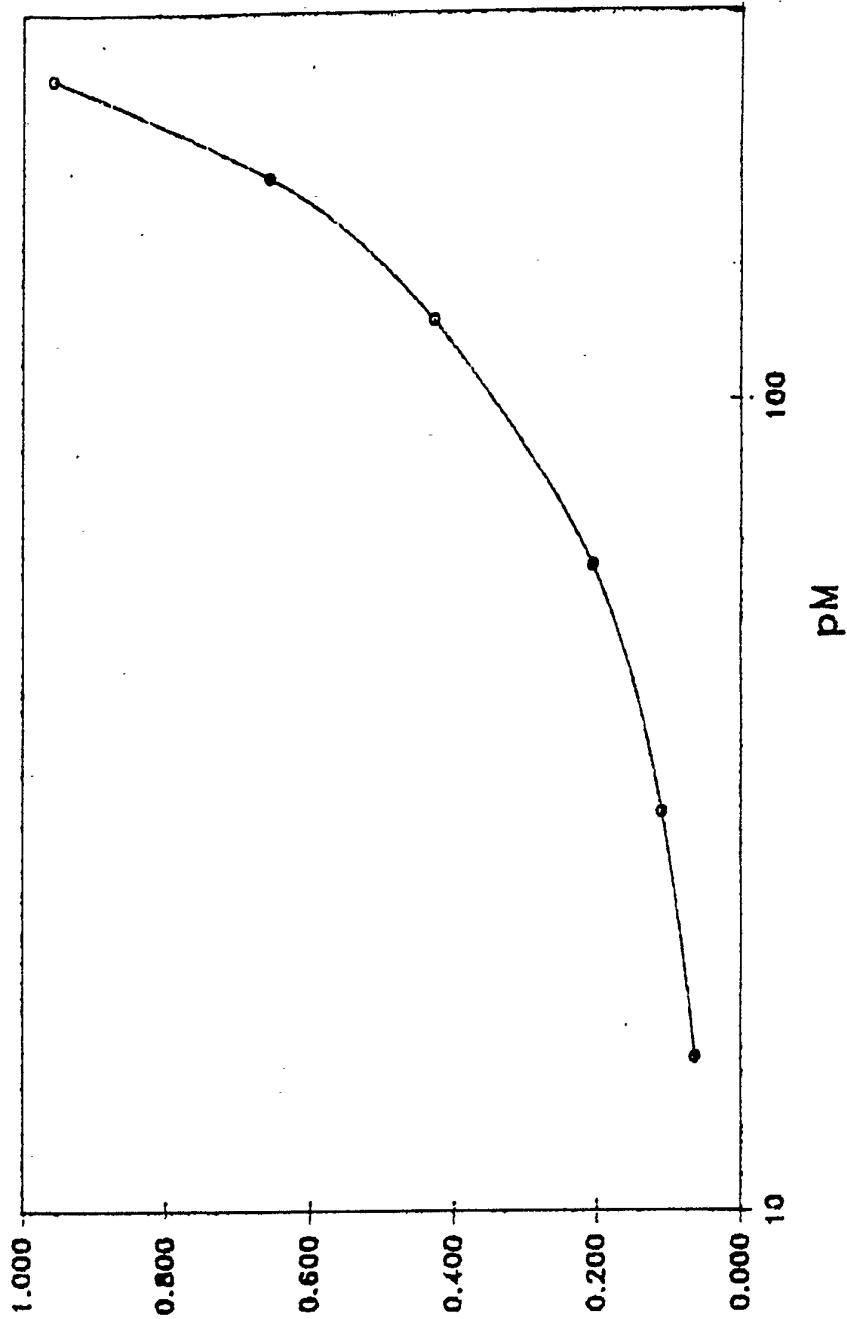
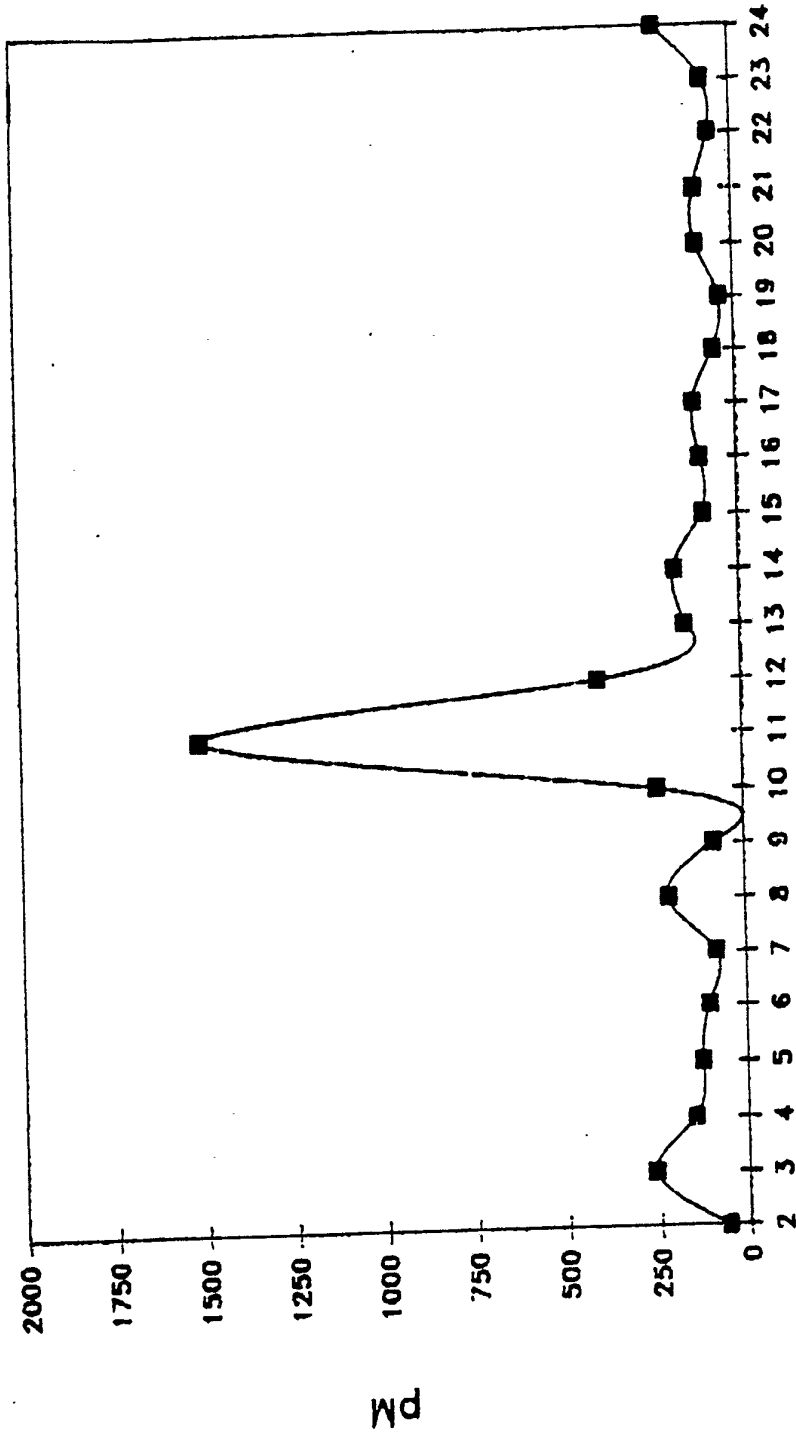


FIG. 2

3/3

TOTAL α EIA
MONTH # 448



CYCLE DAY

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 91/02121

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C 12 Q 1/00 U.S. Cl: 435/7.1		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S.	435/ 4, 7.1, 7.92, 7.94, 28, 188, 805, 810	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	U.S.A. 4,659,678 (FORREST et al) 21 April 1987. See entire document.	1-8
<p>* Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claims or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to be an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ³	
26 April 1991	18 JUN 1991	
International Searching Authority ¹	Signature of Authorized Officer ¹⁹	
ISA/U.S.	<i>Bradley L. Sisson</i> Bradley L. Sisson	