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High-performance liquid chromatography followed by radioimmunoassay for the determination of a luteinizing hormone-releasing hormone analogue, leuprorelin, and its metabolite

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ABSTRACT

A sensitive method for the determination of leuprorelin (TAP-144), a luteinizing hormone-releasing hormone analogue, and its C-terminal metabolite, M-I, in serum and urine has been developed. Leuprorelin and M-I were extracted from serum or urine samples with Sep-Pak C_{18} cartridges, and separated completely by high-performance liquid chromatography and determined by radioimmunoassay using [^{125}I]leuprorelin as the labelled antigen. The detection limit of the method was 0.05 ng/ml for leuprorelin and M-I, and the recovery of the compounds added to serum and urine was over 88% with a coefficient of variation (within-assay) of less than 5%. The method was applied to the determination of leuprorelin and M-I-like immunoreactivity in serum or urine after administration of once-a-month injectable microspheres of leuprorelin acetate (TAP-144-SR) to patients with prostate cancer.

INTRODUCTION

A highly potent luteinizing hormone-releasing hormone (LH-RH) analogue, leuprorelin (D-Leu^6-[des-Gly^{10}-NH_2]-LH-RH-ethylamide, TAP-144) is useful in treating prostate cancer [1–3] and gynecological diseases, such as endometriosis [4–6] and uterine leiomyomata [7,8]. For pharmacokinetic studies of leuprorelin, a radioimmunoassay (RIA) has been used to determine serum and urinary levels [9]. Recently, leuprorelin (5–9)-pentapeptide (M-I), (5–7)-tripeptide (M-II), (1–3)-tripeptide (M-III) and (1–2)-dipeptide (M-IV) were found to be metabolites of leuprorelin in experimental animals given [^{14}C-D-Leu]- or [^{14}C-oxo-Pro]leuprorelin [10]. M-I was found to be highly cross-reactive with a rabbit anti-leuprorelin antibody, which has been used in the RIA for leuprorelin. To examine wheter M-I is actually present in the serum and urine of humans receiving leuprorelin, and to determine accurate concentrations of intact leuprorelin, a method by which leuprorelin and M-I can be determined separately has been sought. This paper describes a method involving a sensitive RIA combined with high-perform-

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ance liquid chromatography (HPLC), which allows the separate determination of leuprorelin and its metabolite M-I in serum and urine.

EXPERIMENTAL

Leuprorelin-related compounds

Leuprorelin and its metabolites, M-I, M-II, M-III and M-IV, were generously supplied from the Research and Development Division, Takeda Chemical Industries (Osaka, Japan). The purities of these peptides, determined by HPLC, were over 96%, and their amino acid sequences were confirmed by amino acid analysis and sequence analysis.

[^{125}]Leuprorelin

Leuprorelin was labelled using the chloramine T method [9].

Antiserum

Rabbit antiserum No. 63-15 [9] was kindly provided by Dr. I. Yamazaki.

Chemicals

Acetic acid, ethanol, methanol (reagent grade), trifluoroacetic acid (TFA, sequence analysis grade) and acetonitrile (HPLC grade) were obtained from Wako (Osaka, Japan). EDTA was purchased from Dojin (Kumamoto, Japan), and goat anti-rabbit γ-globulin and normal rabbit serum were obtained from Daiichi Kagaku (Tokyo, Japan). Bovine serum albumin (RIA grade) was obtained from Sigma (St. Louis, MO, U.S.A.).

HPLC conditions

The HPLC system consisted of a LC-6A solvent-delivery system, a SIL-6A autoinjector, a SPD-6A detector operating at 220 nm, and a C-R4AX chromatographic data analyser (Shimadzu, Kyoto, Japan). An AM-302 octadecylsilyl column (150 mm × 4.6 mm I.D., YMC, Kyoto, Japan) was used, and the column temperature was maintained at 40°C. The elution was performed using an acetonitrile gradient created by mixing two mobile phases: (A) acetonitrile–water (10:90, v/v) with 0.1% TFA, and (B) acetonitrile–water (40:60, v/v) with 0.1% TFA. The gradient consisted of linear segments: 100% A at 0.0 min, 60% A and 40% B at 4.0 min, 40% A and 60% B at 24.0 min, 100% B at 24.1 min, 100% B from 24.1 to 28 min, and 100% A at 28.1 min. The flow-rate was 1.0 ml/min.

Determination of leuprorelin and M-I

Sep-Pak C_{18} cartridges (Millipore, Milford, MA, U.S.A.) were pretreated by the method described elsewhere [11,12]. Serum or urine (1 ml) was diluted with 4 ml of 4% acetic acid in a polypropylene tube, and applied dropwise to the cartridge. The tube was rinsed with 3 ml of 4% acetic acid, and the acetic acid

solution was also applied to the cartridge. The cartridge was washed with 10 ml of water, and leuprorelin and M-I were eluted with 4% acetic acid in 86% (v/v) ethanol. The elutate was dried at 37°C under a nitrogen stream. The residue was dissolved in 0.3 ml of 25% acetonitrile and sonicated for 10 min, then centrifuged at 7000 *g* for 10 min. The supernatant (100 μl) was injected into the HPLC columns every 40 min, and starting 3 min before the retention time of M-I, the eluate from the column was automatically fractionated (2 min per tube) using a Toyo SF-2120 fraction collector (Tokyo, Japan) and dried at 37°C under a nitrogen stream. The residue was dissolved in 0.5 ml of RIA buffer (phosphate-buffered saline containing 1% bovine serum albumin, pH 7.0) and sonicated for 10 min. Leuprorelin- and M-I-like immunoreactivity in the extracts was measured by RIA using ^{125}I-labelled leuprorelin as the labelled antigen [9]. To improve the sensitivity, the amounts of antiserum and the labelled antigen were reduced to 50% of those used in the original method.

RESULTS AND DISCUSSION

A RIA has been used for the determination of serum or urinary leuprorelin in pharmacokinetic studies. Rabbit antiserum No. 63-15 used in the RIA recognizes the C-terminal region of leuprorelin and does not cross-react with LH-RH or TRH [9]. Recently, experiments using [^{14}C-D-Leu]- or [^{14}C-oxo-Pro]leuprorelin showed that leuprorelin is metabolized to shorter peptides, M-I, M-II, M-III and M-IV, in rats and dogs [10]. We therefore studied the influence of these metabolites on the leuprorelin RIA. As shown in Table I, M-II, M-III and M-IV cross-reacted very weakly or hardly at all with the antibody used in the RIA for leuprorelin, whereas the cross-reactivity of M-I was relatively high (70% at the 50% displacement point), indicating that the presence of M-I interfered with the leu-

TABLE I

CROSS-REACTIVITY OF LEUPRORELIN METABOLITES WITH ANTI-LEUPRORELIN ANTIBODY

Peptide sequences: leuprorelin = 5-oxo-Pro-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHC_2H_5 (as acetate); M-I = H-Tyr-D-Leu-Leu-Arg-Pro-NHC_2H_5 (as acetate); M-II = H-Tyr-D-Leu-Leu-OH; M-III = 5-oxo-Pro-His-Trp-OH; M-IV = 5-oxo-Pro-His-OH.

Metabolite	Cross-reactivity (%)[a]
(Leuprorelin)	(100)
M-I	70
M-II	0.009
M-III	<0.001
M-IV	<0.001

[a] Calculated from the concentrations at 50% binding.

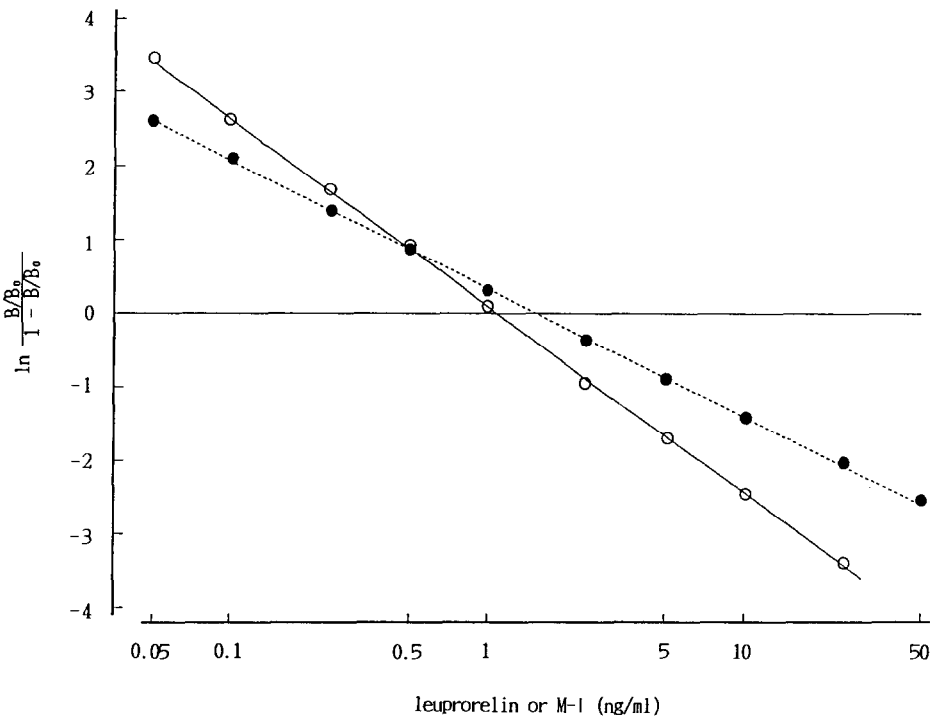

Fig. 1. Calibration curves for leuprorelin (○) and M-I (●) obtained with the RIA using ^{125}I-labelled leuprorelin as the labelled antigen.

prorelin assay. The calibration curve for M-I was not parallel to that for leuprorelin (Fig. 1), which suggested that M-I at concentrations lower than the intersect point was overestimated as leuprorelin, and that M-I at higher concentrations was underestimated. To determine intact leuprorelin and M-I, a method by which the two compounds could be separated by HPLC and determined by RIA utilizing the cross-reactivity of the antibody was investigated.

Prior to HPLC separation, the extraction and concentration of leuprorelin and M-I from serum or urine and the elimination of contaminants were carried out using Sep-Pak C_{18} cartridges. For the separation of leuprorelin and M-I by HPLC, the TFA–acetonitrile–water elution system, which has been widely applied in peptide separation, was used. Being composed of only volatile components, this solvent system was favourable for the drying of the HPLC fractions. The gradient elution system was used to achieve complete separation of leuprorelin and M-I and to eliminate contaminants from serum or urine. Nevertheless, serum and urinary components interfered with UV detection of leuprorelin and M-I at even high concentrations (1000 ng/ml), as shown in Fig. 2. In addition, UV detection limits for authentic leuprorelin and M-I under the present HPLC

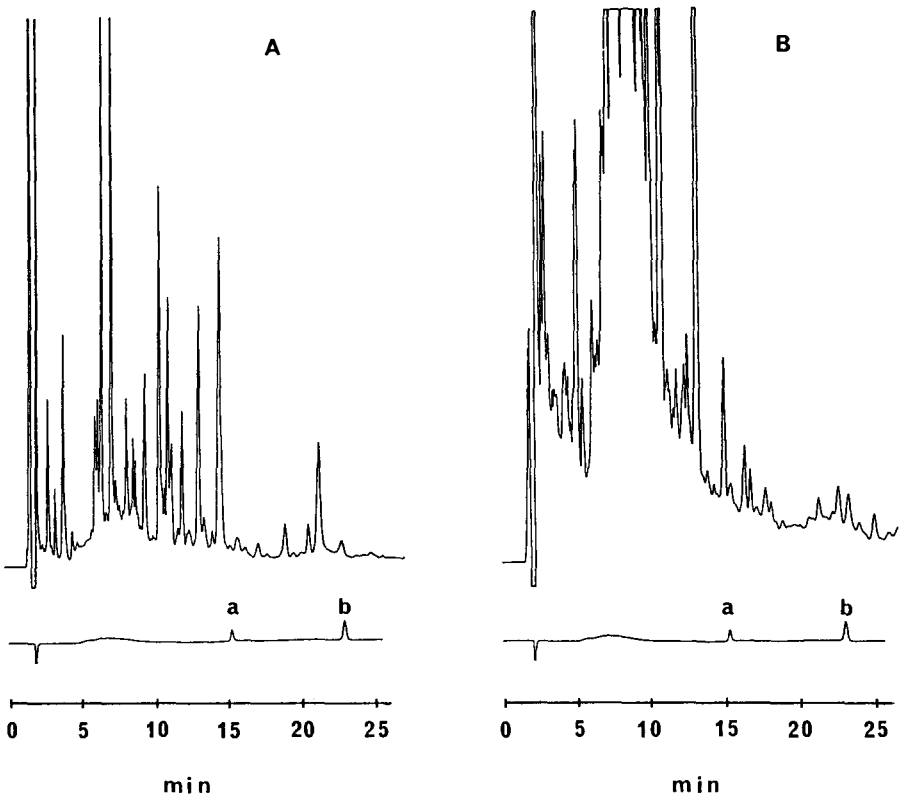

Fig. 2. Chromatograms of extracts of (A) serum and (B) urine, with the chromatograms of (b) leuprorelin and (a) M-I (lower trace) at the same sensitivity. The concentrations of leuprorelin and M-I were 1000 ng/ml each. The absorbance was monitored at 220 nm. Injection volume, 100 μl; detector sensitivity, 0.32 a.u.f.s. Other chromatographic conditions as in the text.

conditions were 1 and 2 ng per injection, respectively, and were too high for serum and urinary levels to be determined, indicating the necessity for an immunological determination method.

Under these HPLC conditions, the retention times of leuprolelin, M-I, M-II, M-III and M-IV were 23.0, 15.3, 21.0, 8.9 and 1.8 min, respectively (Fig. 3). The retention times for M-II, M-III and M-IV were different from those for leuprorelin and M-I, and these metabolites bound very weakly or hardly to the antibody used in the RIA for leuprorelin (Table I). This indicated that M-II, M-III and M-IV did not interfere with the determination of leuprorelin or M-I. The retention times sometimes changed slightly with different columns or eluents, and adsorption of the biological matrix on the HPLC column. Therefore, the retention times had to be ascertained by injecting these peptides prior to sample injection: concentrations of leuprorelin and M-I *ca.* 1000 times higher than those in usually present in serum or urine samples had to be injected for distinct peaks to

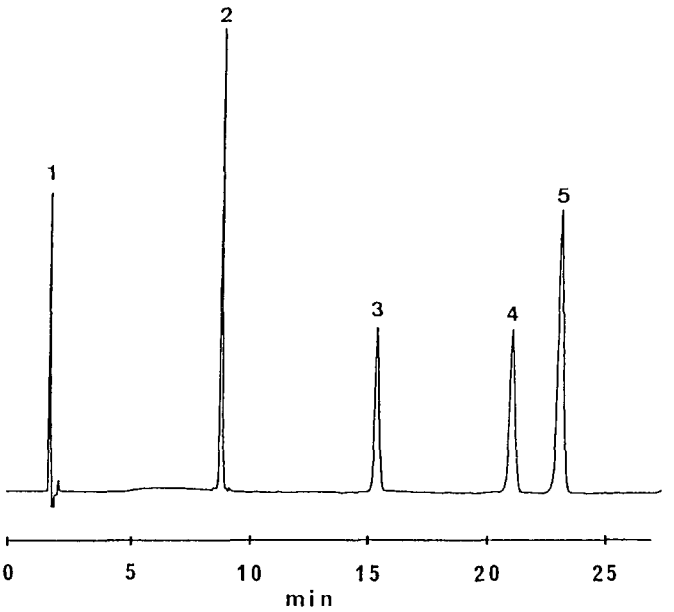

Fig. 3. Chromatographic separation of a mixture of synthetic leuprorelin and its metabolites. Peaks: 1 = M-IV; 2 = M-III; 3 = M-I; 4 = M-II; 5 = leuprorelin. The concentrations were 250 ng/ml for M-III and M-IV, and 1000 ng/ml for M-I, M-II and leuprorelin. The absorbance was monitored at 220 nm. Injection volume, 100 μl; detector sensitivity, 0.04 a.u.f.s. Other chromatographic conditions as in the text.

be detected. Therefore, careful attention was required to avoid the retention of such high concentrations of solution in the chromatographic lines. No leuprorelin- and M-I-like immunoreactivities were detected in extracts of serum and urine obtained prior to leuprorelin injection (Fig. 4A and C, and Fig. 5A and C), which demonstrates that the retention of injected peptides in the HPLC line was negligible and that RIA background values were appropriately low. The immunoreactivities were detected in extracts from samples collected 24 h after the injection (Fig. 4B and D, and Fig. 5B and D). The positions of immunoreactive fractions coincided with the retention times of leuprorelin and M-I, suggesting that the influence of biological components on the retention times of both peptides could be neglected.

For the determination of M-I, ^{125}I-labelled leuprorelin was again used as the labelled antigen to avoid the complexity caused by the use of two labelled antigens. In practice, a very highly sensitive assay for M-I, as shown in Fig. 1, was achieved using labelled leuprorelin instead of labelled M-I. The detection limits for leuprorelin and M-I were 0.05 ng/ml for serum and urine. Serum and urine spiked with leuprorelin and M-I at the concentrations of 0.5 and 2.5 ng/ml were assayed repeatedly, to determine the recovery of leuprorelin and of M-I and to calculate within-assay precision (Table II). The mean recovery was over 88% and the coefficient of variation (within-assay) was less than 5%.

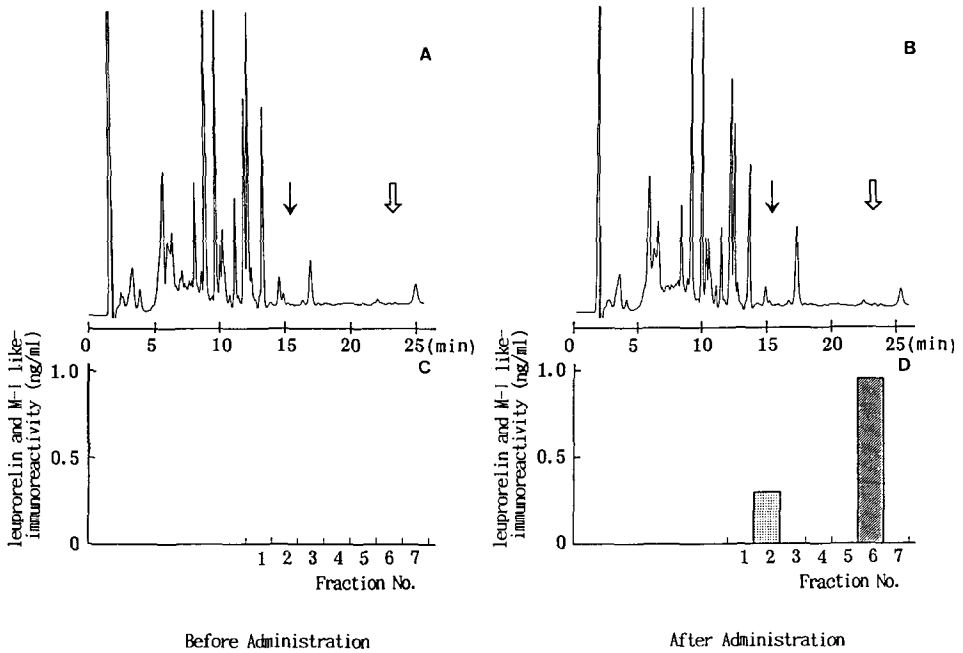

Fig. 4. Chromatograms (A and B) and the leuprorelin- and M-I-like immunoreactivity levels (C and D) in serum samples obtained before and 24 h after intramuscular injection of leuprorelin. The arrows indicate the retention times of leuprorelin (⇩) and M-I (↓), respectively.

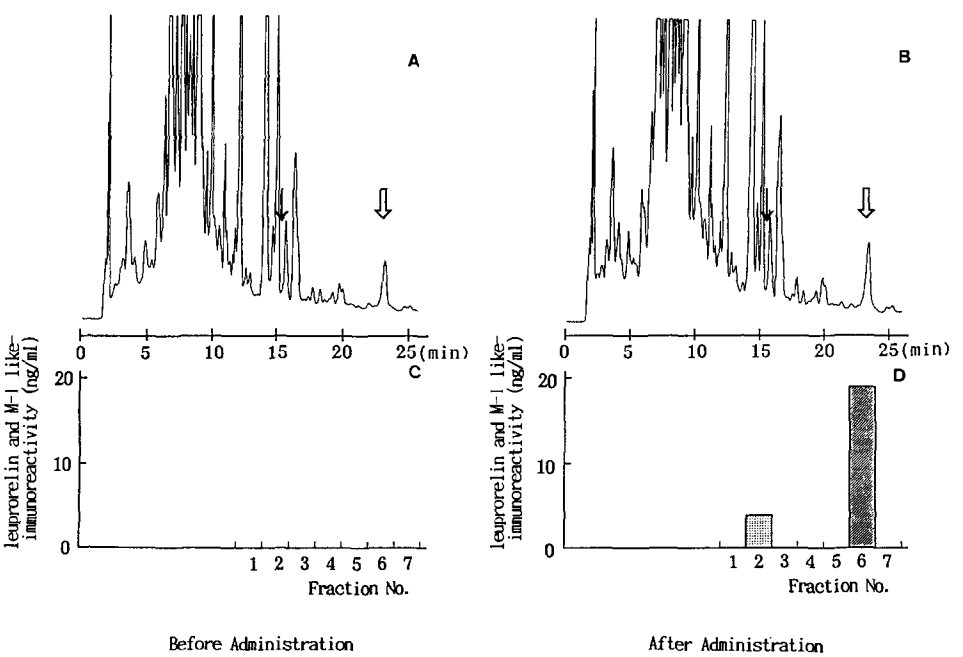

Fig. 5. Chromatograms (A and B) and the leuprorelin- and M-I-like immunoreactivity levels (C and D) in urine samples obtained before and 24 h after intramuscular injection of leuprorelin. The arrows indicate the retention times of leuprorelin (⇩) and M-I (↓), respectively.

TABLE II

RECOVERIES OF LEUPRORELIN AND M-I ADDED TO DRUG-FREE SERUM AND URINE, AND THE REPRODUCIBILITY OF THE METHOD

Leuprorelin and M-I were added to drug-free serum and urine at the concentrations 0.50 and 2.50 ng/ml and determined by the present method using 1 ml of serum and urine.

Compound	Concentration spiked (ng/ml)	Concentration found (mean ± S.D., n = 7) (ng/ml)	Precision (C.V.) (%)	Recovery (%)
In serum				
Leuprorelin	0.50	0.449 ± 0.012	2.7	89.8
	2.50	2.401 ± 0.079	3.3	96.0
M-I	0.50	0.444 ± 0.015	3.4	88.8
	2.50	2.381 ± 0.078	3.3	95.2
In urine				
Leuprorelin	0.50	0.476 ± 0.022	4.7	95.2
	2.50	2.450 ± 0.118	4.8	98.0
M-I	0.50	0.467 ± 0.021	4.6	93.4
	2.50	2.453 ± 0.110	4.5	98.0

Serum and urinary leuprorelin- and M-I-like immunoreactivity after intramuscular injection of once-a-month injectable microspheres of leuprorelin (TAP-144-SR, 3.75 mg as leuprorelin acetate) [13,14] in patients with prostate cancer were determined (Tables III and IV). Leuprorelin- and M-I-like immunoreactivity were detected in serum and urine within a short time after the injection. Serum leuprorelin levels tended to be higher than serum M-I levels, and the ratio of leuprorelin to M-I in serum was higher than that in urine. Leuprorelin was detected in serum even four weeks after the injection.

A HPLC–RIA method similar to the present one has been reported for buserelin, a LH-RH agonist, and used to determine intact buserelin and its C-terminal metabolite for detailed evaluation of buserelin pharmacokinetics [15]. In that study, buserelin (not a formulation intended for sustained release) was administered intravenously or intranasally, therefore serum levels after the administration were relatively high, and the elimination from the blood circulation was fast. In our study, TAP-144-SR is designed to allow an effective level of drug to be maintained for one month after a single administration [13,14]. Serum and urinary leuprorelin- and M-I-like immunoactivity levels can be determined by the present method for four weeks after a single intramuscular injection of a therapeutic dose of TAP-144-SR to patients with prostate cancer. Therefore, the present method is considered to be useful in pharmacokinetic evaluation of TAP-144-SR. Besides, metabolites other than M-I could not be determined because antibodies against these metabolites have not yet been obtained.

TABLE III

SERUM LEUPRORELIN- AND M-I-LIKE IMMUNOREACTIVITY LEVELS AFTER ADMINISTRATION OF TAP-144-SR TO A PATIENT WITH PROSTATE CANCER

Patient K.M.; dose 3.75 mg as leuprorelin acetate.

Time after administration	Leuprorelin[a] (ng/ml)	M-I[a] (ng/ml)
Before	N.D.[b]	N.D.
0.5 h	5.23	N.D.
1 h	12.0	0.15
2 h	14.0	0.73
3 h	9.54	0.86
6 h	5.19	0.77
1 day	0.95	0.29
2 days	0.18	0.07
3 days	0.16	0.09
1 week	0.08	N.D.
2 weeks	0.06	N.D.
4 weeks	0.13	0.05

[a] Data are expressed as acetate.
[b] N.D. < 0.05 ng/ml.

TABLE IV

URINARY LEUPRORELIN- AND M-I-LIKE IMMUNOREACTIVITY LEVELS AFTER ADMINISTRATION OF TAP-144-SR TO A PATIENT WITH PROSTATE CANCER

Patient H.I.; dose, 3.75 mg as leuprorelin acetate.

Time after administration (days)	Leuprorelin[a] (ng/ml)	M-I[a] (ng/ml)
Before	N.D.[b]	N.D.
1	19.2	4.12
2	6.72	4.97
3	2.11	1.13
5	1.21	0.93
7	2.20	1.60
10	0.84	0.98
15	1.43	0.85
20	1.89	0.89
25	2.53	1.79
29	2.13	1.74

[a] Data are expressed as acetate.
[b] N.D. < 0.05 ng/ml.

Immunoassays have been effectively used for the determination of proteins and peptides in biological fluids. However, when the amino acid sequence of a peptide or protein closely resembles that of others, it is difficult to produce an antibody that differentiates one from the others. In addition, non-specific reaction and inhibition with the biological matrix in immunoassays always have to be considered. In such cases, immunoassays combined with HPLC, such as the present method, may be useful for improving the specificity and reducing the adverse reactions.

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REFERENCES

1 G. W. Chodak, *Urology*, 33 (1989) 42.
2 T. Mazzei, M. Eandi, E. F. Reali, L. Fioretto, R. Bartoletti, M. Rizzo, G. Calabro and P. Periti, *Drugs Exp. Clin. Res.*, 15 (1989) 373.
3 R. Sharifi, Y. Soloway, R. J. Correa, Jr., A. G. Glass, P. D. Guinan, M. B. Garnick, L. M. Glode, J. A. Smith and B. S. Stein, *J. Urol.*, 143 (1990) 68.
4 A. J. Friedman and R. L. Barbieri, *Curr. Probl. Obstet. Gynecol. Fertil.*, 11 (1988) 209.
5 I. S. Tummon, M. E. Pepping, Z. Binor, E. Radwanska and W. P. Dmowski, *Fertil. Steril.*, 51 (1989) 390.
6 M. E. Rivlin, J. D. Miller, R. P. Kruegger, R. B. Patel and J. D. Bower, *Obstet. Gynecol.*, 75 (1990) 532.
7 W. D. Schlaff, E. A. Zerhouni, J. A. M. Huth, J. Chen, M. D. Damewood and J. A. Rock, *Obstet. Gynecol.*, 76 (1989) 856.
8 A. J. Friedman, B. B. Benacerraf, D. Harrison-Atlas, R. Gleason, R. L. Barbieri and I. Schiff, *Fertil. Steril.*, 51 (1989) 251.
9 I. Yamazaki and H. Okada, *Endocrinol. Jpn.*, 27 (1980) 593.
10 I. Naeshiro, T. Kondo, M. Mitani, K. Yoshida, T. Kobayashi, T. Kimura, H. Shimomura and S. Tanayama, *Jpn. Pharmacol. Ther.*, 18 (Suppl. 3) (1990) S-545.
11 F. Rosmalen, A. Tan, H. S. Tan and T. Benraad, *Clin. Chim. Acta*, 165 (1987) 331.
12 N. Suzuki, H. Matsumoto, C. Kitada, T. Masaki and M. Fujino, *J. Immunol. Methods*, 118 (1989) 245.
13 Y. Ogawa, H. Okada, T. Heya and T. Shimamoto, *J. Pharm., Pharmacol.*, 41 (1988) 439.
14 H. Okada, T. Heya, Y. Ogawa and T. Shimamoto, *J. Pharmacol. Exp. Ther.*, 244 (1988) 744.
15 L. Kiesel, J. Sandow, K. Bertges, G. Jeraber-Sandow, H. Trabant and B. Runnebaum, *J. Clin. Endocrinol. Metab.*, 68 (1989) 1167.