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Short communication

Determination of taltirelin, a new stable thyrotropin-releasing hormone analogue, in human plasma by high-performance liquid chromatography turbo-ionspray ionization tandem mass spectrometry

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Abstract

A rapid, selective and sensitive assay of taltirelin, a novel thyrotropin-releasing hormone analogue, in human plasma has been developed. This method is based on a rapid sample preparation and high-performance liquid chromatography (HPLC) turbo-ionspray ionization tandem mass spectrometry (MS-MS). Analytes were purified from human plasma by SPE cartridge and separated by gradient HPLC. Turbo-ionspray ionization and MS-MS analyses were carried out by PE-Sciex API 3000 tandem mass spectrometer. Taltirelin was separated from its metabolite (acid form) on a semi-micro ODS column in methanol – 0.1% (v/v) formic acid. The selected reaction monitoring by precursor → product ion combination of m/z 406 → 264, was used for determination of taltirelin. The linearity was confirmed in the concentration range of 17–4137 pg/ml in human plasma, and the precision of this assay, expressed as a relative deviation, was less than 9.8% over the entire concentration range with adequate assay accuracy. The results obtained by the HPLC-MS-MS method correlated well with those of the radioimmunoassay method reported previously. Therefore, the HPLC-MS-MS method is useful for the determination of taltirelin with sufficient selectivity and sensitivity on pharmacokinetic studies in human.

Keywords: Taltirelin; Thyrotropin-releasing hormone; HPLC-MS-MS; SPE; Cross-validation

1. Introduction

Taltirelin, (−)-*N*-[(*S*)-hexahydro-1-methyl-2,6-dioxo-4-pyrimidinyl carbonyl]-L-histidyl-L-prolinamide tetrahydrate (Fig. 1), is a new thyrotropin-releasing hormone (TRH) analogue developed by Tanabe Seiyaku [1]. It has various central

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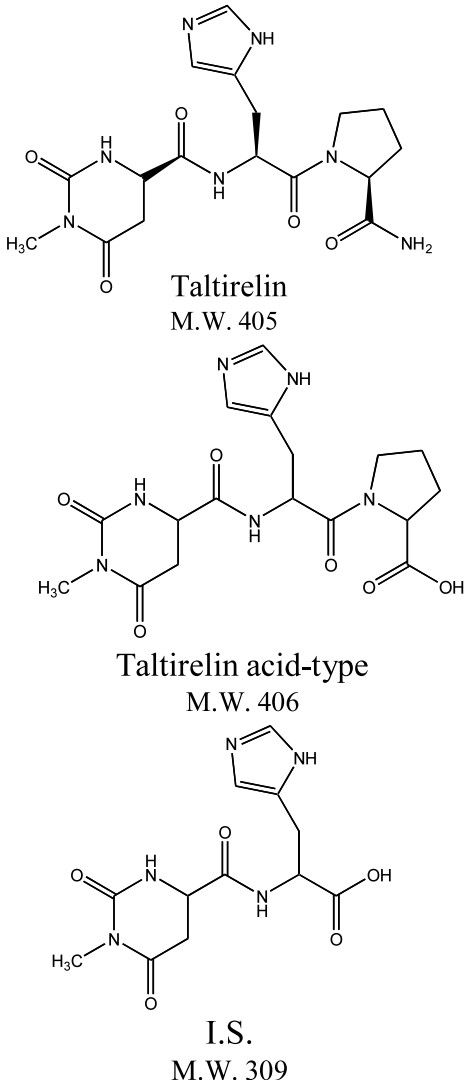

Fig. 1. Chemical structures of taltirelin, its acid-type and the I.S.

nervous system (CNS) activities 30–100-fold more potent and about 8-fold longer than TRH [2]. In contrast, its thyroid stimulating hormone releasing activity is only 1/50th of that of TRH [1], indicating a successful dissociation between the CNS and hormonal actions. Also, taltirelin produced dose-dependent increases in the amplitudes of mono- and poly-synaptic reflex potentials and withdrawal flexor reflexes [3].

The clinical dose of taltirelin is as low as 5 mg twice a day, therefore, it is necessary to develop a sensitive assay for taltirelin in human plasma to investigate the pharmacokinetics of taltirelin in human. A radioimmunoassay (RIA) method was developed to determine taltirelin and its metabolite, taltirelin acid-type (Fig. 1) in human plasma and urine [4]. The RIA method needs about 3 days for measurement of taltirelin and taltirelin acid-type. The inter-assay variations of the RIA method for taltirelin were 15.5% at 40 pg/ml, 3.5% at 400 pg/ml and 11.2% at 4000 pg/ml in human plasma. The limit of quantification for taltirelin was 20 pg/ml in human plasma. For calibration curve, this method needs each blank plasma of patient. It is very difficult to obtain blank plasma from patients, especially infants. Therefore, different rapid and sensitive determination method is required.

The HPLC-MS-MS method has recently, been demonstrated to be a useful technique for a quantitative determination of drugs and metabolites in biological fluids. An angiotensin-converting enzyme inhibitor, imidapril and its active metabolite in human plasma were determined by HPLC-MS-MS [5]. The limits of quantification were 0.2 ng/ml. Buprenorphine, norbprenorphine and buprenorphine–glucuronide in human plasma were also determined simultaneously by HPLC-MS-MS [6]. The limits of quantification were 0.1 ng/ml for the three analytes. The high selectivity and sensitivity by the MS-MS detection with the multiple reaction monitoring (MRM) make it possible to determine the amount of drugs at very low concentrations; 0.5 ng/ml of cyclic peptide [7], 0.2 ng/ml of oxycodone [8], 0.5 ng/ml of β3-agonist [9]. The reduction of sampling volume reduces the stress to patients in clinical studies. The cross-validations were often performed between new HPLC-MS-MS methods and established RIA methods, and the HPLC-MS-MS methods using ionspray interface has good sensitivity and accuracy [10–12].

This paper describes the rapid, selective, and sensitive determination of taltirelin in human plasma by HPLC-MS-MS with MRM after obtaining the validation and cross-validation data between the HPLC-MS-MS and the RIA methods.

2. Experimental

2.1. Reagents and materials

Taltirelin and its main metabolite, acid-type (Fig. 1) were synthesized by Tanabe Seiyaku Co. Ltd (Osaka, Japan). *N*-[(*S*)-hexahydro-1-methyl-2,6-dioxo-4-pyrimidinylcarbonyl]-L-histidine synthesized by Tanabe Seiyaku Co. Ltd, was used as an internal standard (I.S.). Sodium carbonate and formic acid were of reagent grade from Katayama Chemical Inc. (Osaka, Japan). Methanol was of HPLC grade from Fisher Scientific (PA, USA). OASIS HLB 3 cc (60 mg) extraction cartridges based on co-polymer (divinyl benzene–*N*-vinylpyrrolidone), were obtained from Waters (Milford, MA). Millex-HV filters (0.45 μm) were obtained from Millipore Co. (MA, USA). The drug-free human heparinized plasma was obtained from healthy volunteers who provided informed consent and were paid for their participation.

2.2. Instrumentation

A API 3000 (PE-Sciex, CA, USA) tandem mass spectrometer equipped with a TurboIonSpray interface and a Agilent 1100 HPLC system (Agilent Technologies Inc., USA) were used for all HPLC-MS-MS analyses. HPLC separations were performed by using a Inertsil ODS-2 150 × 2.1 mm I.D., 5 μm particle size (GL-Science Inc. Tokyo, Japan), a Symmetry C_{18} 150 × 2.1 mm I.D., 5 μm particle size (Waters Corporation, MA, USA), a CAPCELL PAK UG-120 150 × 2.0 mm I.D., 5 μm particle size (Shiseido Co. Ltd, Tokyo, Japan), a TSKgel ODS-80Ts 150 × 2.0 mm I.D., 5 μm particle size (Tosoh Corporation, Tokyo, Japan), a Cosmosil 5C18-AR-300 150 × 2.0 mm I.D., 5 μm particle size (nacalai tesque Co. Ltd, Kyoto, Japan) and a DAISOPAK SP-120-30DS-BP 150 × 2.0 mm I.D., 5 μm particle size (Daiso Co. Ltd, Osaka, Japan). Pump 11 single syringe (Harvard Apparatus, MA, USA) was used for sample injection.

2.3. Standard solutions

The stock standard solution of taltirelin (1 mg/ml) was prepared with distilled water. This solution was further diluted with distilled water to the working standard solutions at given concentrations for validation and calibration. The stock I.S. solution (1 mg/ml) was also prepared with distilled water. The working I.S. solution of 50 ng/ml was prepared by dilution of the stock I.S. solution with methanol – 0.1% (v/v) formic acid (3:37, v/v). All stock solutions were stored at −20 °C.

2.4. Sample preparation

A 500 μl aliquot of plasma sample was pipetted into a glass test tube, and 50 μl of the mobile phase A and 1 ml of 0.2 M sodium carbonate were added. The mixture was applied to an OASIS HLB cartridge, which was previously conditioned with 1 ml of methanol, 1 ml of distilled water, and 1 ml of 0.2 M sodium carbonate, respectively. The cartridge was washed with 2 ml of distilled water. Taltirelin retained in the cartridge were eluted with 1 ml of methanol into a disposable glass test tube, and evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved in 250 μl of the mobile phase A (Section 2.5) contained 12.5 ng of I.S. A 25 μl of the solution was analyzed by HPLC-MS-MS.

2.5. HPLC-MS-MS conditions

Chromatographic separation was carried out using a DAISOPAK 150 × 2.0 mm I.D., 5 μm particle size, at a column temperature of 40 °C. Methanol – 0.1% (v/v) formic acid (3:37, v/v) as the mobile phase A (A) and methanol – 0.1% (v/v) formic acid (9:1, v/v) as the mobile phase B (B) were used at flow rate of 0.2 ml/min. Gradient elution was performed as follows: 100% A for 3 min, linear decrease to 0% A in 1 min, 3 min hold at 0% A, 0.1 min step increase to 100% with 12.9 min equilibration before the following injection (total run time: 20 min). The temperature of the sample cooler in auto-sampler was set at 10 °C.

Ionization of analyte was carried out using the following settings of the turbo–ionspray interface:

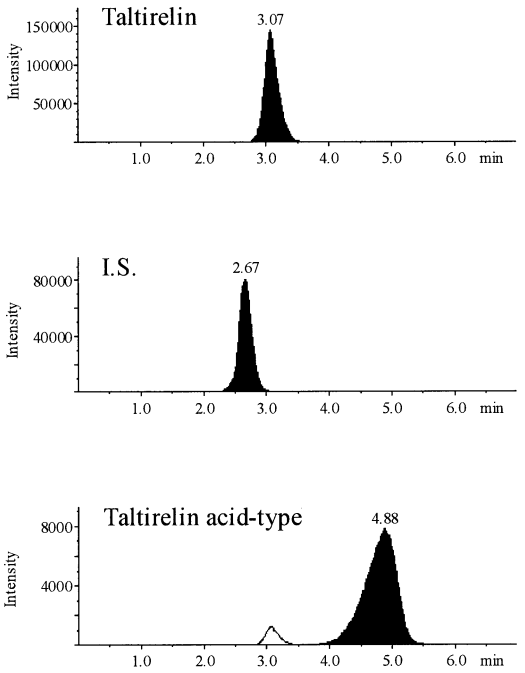

Fig. 2. HPLC-MS chromatograms of taltirelin, the I.S. and taltirelin acid-type.

ionization mode, positive; source temperature and flow rate, 475 °C and 7 l/min, respectively; ion-spray voltage, 5000 V; nebulizer gas (pure air) and curtain gas (nitrogen) setting, 14 and 12, respectively.

Tandem mass spectrometric analysis was performed using nitrogen as collision gas (setting, 4). The m/z 406 → m/z 264 transition (collision energy, −80 eV) was monitored for taltirelin and the m/z 310 → m/z 264 was monitored for I.S.

2.6. Validation tests

2.6.1. Linearity and calibration curve

Standards for linearity at nine concentrations (0, 17, 41, 83, 165, 414, 827, 1655 and 4137 pg/ml in plasma) were prepared and assayed. To determine the precision of the slope of the calibration curve, each calibration standard at six concentrations (0, 17, 41, 165, 827 and 4137 pg/ml in plasma, intra-day; 0, 18, 44, 177, 883 and 4413 pg/ml in plasma, intra-day) were also prepared and assayed.

2.6.2. Specificity and interference

Chromatograms of the sample prepared with human blank plasma were visually inspected for peaks from endogenous sources which might correspond to taltirelin and I.S. peaks.

2.6.3. Accuracy and precision

Samples at each of four concentrations (17, 41, 414 and 4137 pg/ml in plasma, $n = 6$, intra-day; 18, 44, 441 and 4413 pg/ml in plasma, $n = 6$, inter-day) were prepared and assayed to determine the intra- or inter-day accuracy expressed as relative error, and precision as coefficient of variation (C.V.).

2.6.4. Cross-validation

Samples at each of three concentrations (100, 500 and 2500 pg/ml in plasma, $n = 6$) were prepared and assayed to determine taltirelin by the RIA and the HPLC-MS-MS methods. The found concentrations obtained by the HPLC-MS-MS method were compared with that by the RIA method, and the correlation between the methods was demonstrated.

2.7. Radioimmunoassay method

The RIA method was developed to determine taltirelin and taltirelin acid-type in human plasma and urine, previously [4]. This method was used for cross-validation between the RIA and the HPLC-MS-MS methods in human plasma.

3. Results and discussion

3.1. HPLC-MS-MS conditions

3.1.1. High-performance liquid chromatography

The molecular weights of taltirelin and its main metabolite, acid-type were 405 and 406, respectively. Using HPLC-MS-MS, isotopic ion of taltirelin disturbed determination of acid-type. Consequently, it is necessary to separate taltirelin and acid-type completely on HPLC. Six types of HPLC columns (DAISOPAK, Inertsil ODS-2, Symmetry C_{18}, CAPCELL PAK UG-120, TSKgel ODS-80Ts, Cosmosil 5C18-AR-300) were investigated for separation. Using DAISOPAK which

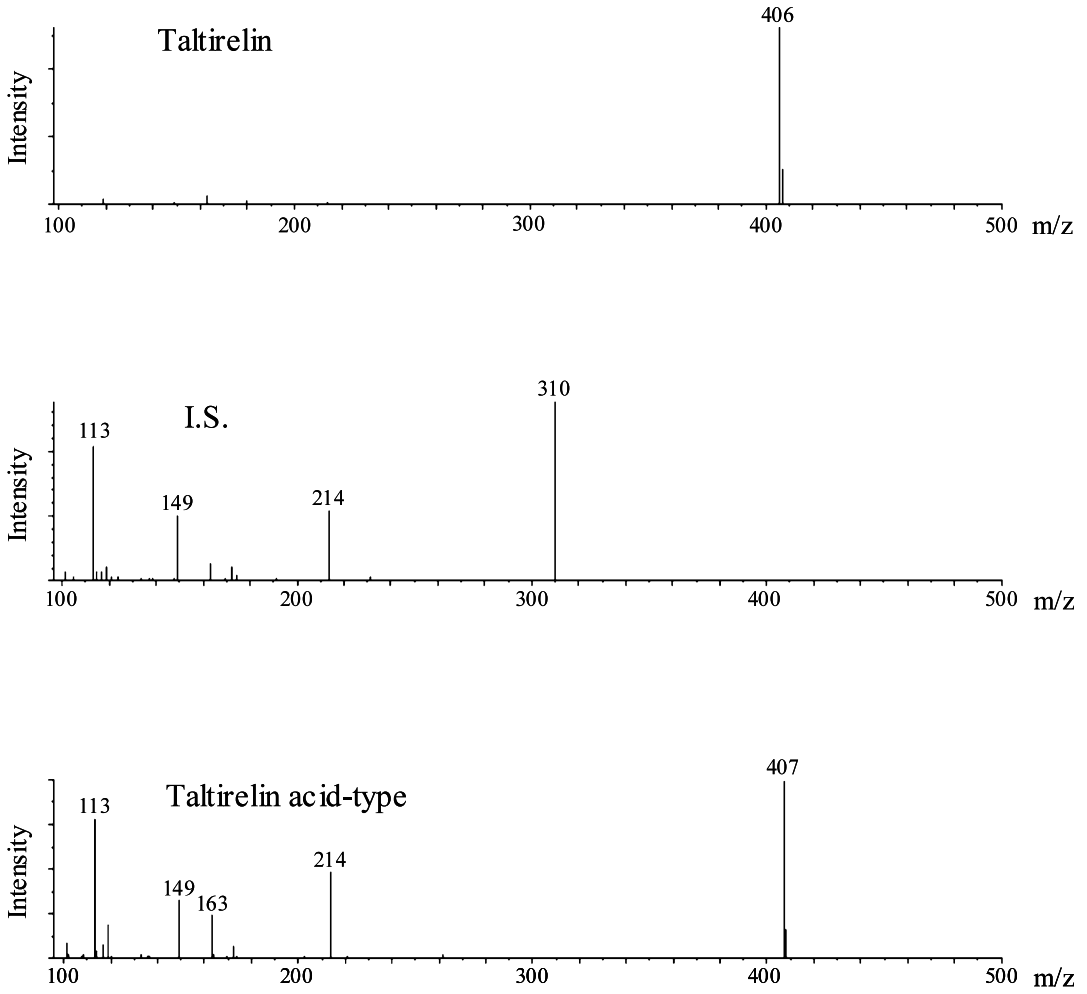

Fig. 3. Mass spectra of taltirelin, the I.S. and taltirelin acid-type.

can separate hydrophilic compounds on HPLC, though the peak of acid-type was broad, taltirelin and acid-type were completely separated with 0.1% (v/v) formic acid–methanol (Fig. 2). Because DAISOPAK has large cavity size comparison with other ODS columns. Other columns could not separate taltirelin and acid-type completely.

3.1.2. MS-MS

Each compound was first directly introduced in a mass spectrometer using the syringe pump and the turbo–ionspray ionization interface to get individual mass spectra (Fig. 3). Parameters such as source temperature, ionspray voltage, nebulizer and curtain gases were optimized in order to obtain much stronger intensity of the protonated molecules. The protonated molecule $[M+H]^+$ was identified at m/z 406 and 310 for taltirelin and I.S., respectively. The product–ion spectra of taltirelin and I.S. were acquired with these protonated molecules as precursors (Fig. 4). The most suitable collision energy and collision gas pressure were set by observing the response of the fragment ion peak. The product ions mass spectrum of taltirelin

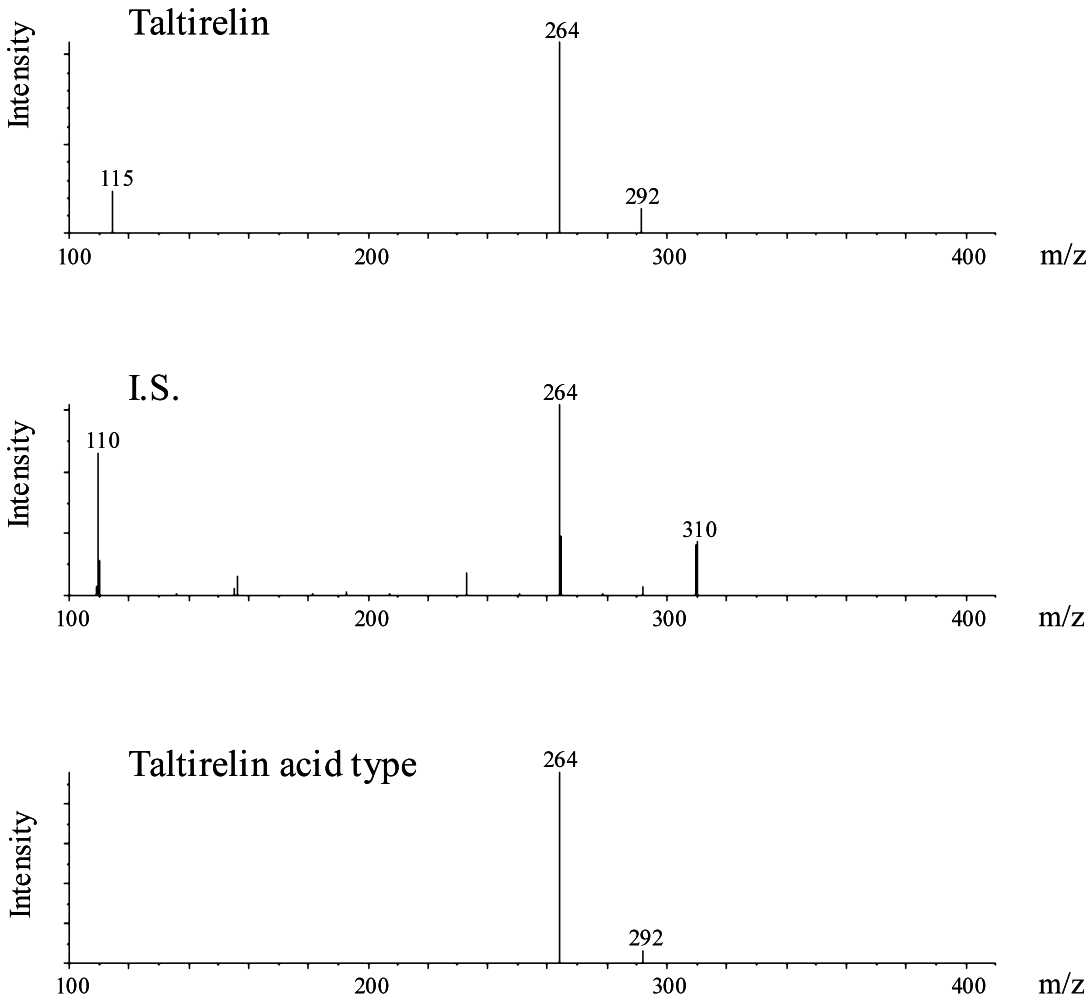

Fig. 4. Product–ion spectra of taltirelin, the I.S. and taltirelin acid-type.

Table 1
Recoveries of taltirelin and I.S. from human plasma by SPE method

Nominal concentration (ng/ml)	Recovery (%)	
	Taltirelin[a]	I.S.
Taltirelin		
100	94.9±8.3	–
500	91.6±2.7	–
2500	91.4±2.0	–
I.S.		
5000	–	0.0

[a] Mean±S.D. ($n = 6$).

and I.S. showed the same predominant fragment ion at m/z 264. The same pattern was found in a case of taltirelin and I.S.; therefore, such kinds of fragment ions were selected for the determination, at m/z 264 for taltirelin and I.S.

3.2. Sample preparation

A highly sensitive and reproducible analytical method for biological samples needs suitable pretreatments if low selective detection system was used. The technique using MS-MS has high selectivity and sensitivity, and can simplify the

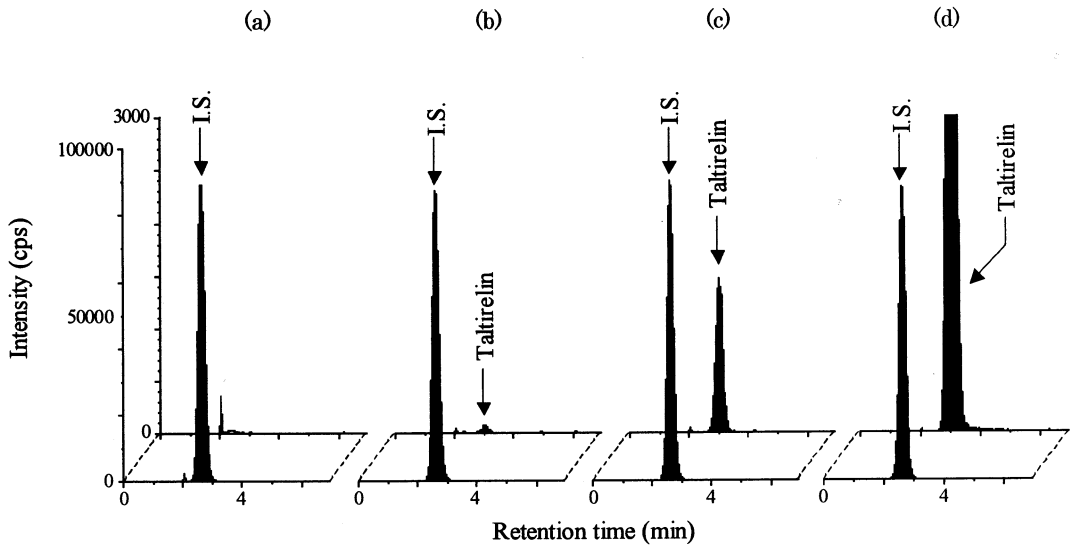

Fig. 5. Representative HPLC-MS-MS chromatograms of blank plasma (a) with the I.S. and spiked plasma with 20 (b) 400 (c) and 4000 pg/ml plasma (d) of taltirelin.

Table 2
Intra-day precision and accuracy of taltirelin spiked in human plasma by HPLC-MS-MS

Nominal concentration (pg/ml)	Found concentration[a] (pg/ml)	Accuracy (error %)	C.V. (%)
16.5	17.1 ± 0.0	3.8	0.0
41.4	41.0 ± 2.4	−0.9	5.7
413.7	423.1 ± 40.7	2.3	9.6
4136.9	4034.2 ± 286.8	−2.5	7.1

[a] Mean ± S.D. ($n = 6$).

Table 3
Inter-day precision and accuracy of taltirelin spiked in human plasma by HPLC-MS-MS.

Nominal concentration (pg/ml)	Found concentration[a] (pg/ml)	Accuracy (error %)	C.V. (%)
17.7	17.7 ± 1.7	−0.1	9.7
44.1	45.8 ± 1.7	3.8	3.7
441.3	437.3 ± 43.0	−0.9	9.8
4412.7	4619.3 ± 325.7	4.7	7.1

[a] Mean ± S.D. ($n = 6$).

preparation procedure to the greatest extent possible.

The OASIS HLB solid phase extraction cartridge based on co-polymer (divinyl benzene–*N*-vinylpyrrolidone) retains water strongly and resists against drying. It was chosen to isolate taltirelin from human plasma. The mixture of the plasma sample, the mobile phase A and the buffer was charged into the previously conditioned OASIS HLB cartridge. After washing the cartridge, taltirelin was isolated by elution with methanol and the eluate was evaporated to dryness. The residue was

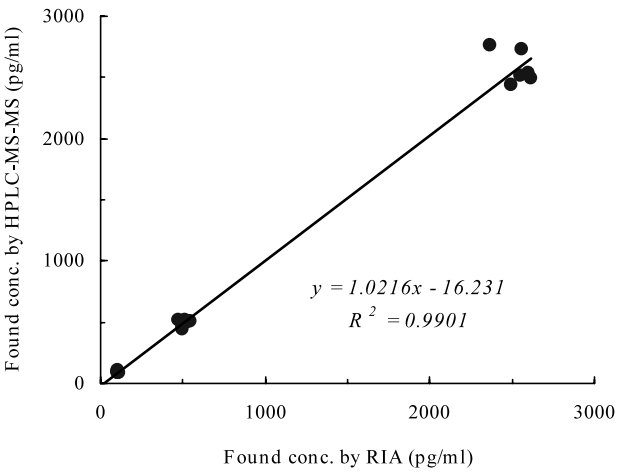

Fig. 6. Correlation between the RIA and HPLC-MS-MS methods.

dissolved in the mobile phase A contained I.S. Because I.S., which has a carboxylic group, has low recovery from OASIS HLB with this preparation procedure, I.S. was added after the solid phase extraction (Table 1). These procedures make it possible to extract taltirelin from human plasma rapidly and quantitatively. Comparison with the RIA method, this preparation method could be reduced measurement time for taltirelin in human plasma.

The stability of the standard solution at −20 °C was assessed. Taltirelin was stable for approximately 3 months. In the auto-sampler at 10 °C, taltirelin was stable for 24 h. The recoveries of taltirelin from human plasma through this extraction procedure were 91.4–94.9% in the range of 100–2500 pg/ml plasma (Table 1).

3.3. Validation

3.3.1. Linearity and calibration curves

Good linearity was observed over the concentration range of 17–4137 pg/ml in human plasma ($r^2 = 0.9999$). Typical equation ($1/x^2$ weighed regression analysis) was used for calibration curves. The C.V. ($n = 7$) of the slopes calculated with calibration curve data was 9.2% for taltirelin, showing good reproducibility.

3.3.2. Selectivity and specificity

HPLC-MS-MS in the MRM mode provides a highly selectivity for the determination of drugs in biological samples. The representative MRM chromatograms of blank plasma and spiked plasma samples are shown in Fig. 5. No endogenous sources of interference were observed at the retention time of the analyte.

3.3.3. Accuracy, precision and limit of quantification

The intra- and inter-day accuracy and precision are assessed in Tables 2 and 3. The error percentage of taltirelin ranged from −2.5 to 3.8% for intra-day and −0.9 to 4.7% for inter-day. The C.V. of taltirelin ranged from 0.0 to 9.6% for intra-day and 3.7 to 9.8% for inter-day. The limit of quantification was established at 17 pg/ml in human plasma on the basis of the accuracy of the determinations at this concentration (deviation from the nominal value within 20%). Comparison with the RIA method, the HPLC-MS-MS method obtained better precision at the low concentrations and the same quantitative limit.

3.3.4. Cross-validation

In order to evaluate the HPLC-MS-MS method, the present method, the RIA method was applied to determination of human plasma samples prepared for the cross-validation. The found concentrations obtained by the HPLC-MS-MS method were compared with that by the RIA method, and the correlation between the methods was demonstrated (Fig. 6). Good correlation between the RIA method and the HPLC-MS-MS was found.

4. Conclusions

The HPLC-MS-MS method described in this paper permits the rapid, selective and sensitive

determination of taltirelin in human. The method is suitable for use in clinical studies, because it is not necessary to prepare blank plasma obtained from patients to determine taltirelin. The results obtained by the HPLC-IS-MS-MS method correlated well with those of the RIA method reported previously. These analytical validation and cross-validation for RIA demonstrate that this method allows the rapid and sensitive determination of taltirelin in human plasma, compared with the RIA method. Therefore, the HPLC-MS-MS method is useful for the determination of taltirelin with sufficient selectivity and sensitivity on pharmacokinetic studies in human.

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