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Highly sensitive liquid chromatography-tandem mass spectrometry method for quantification of a new bone anabolic agent, TAK-778, in human serum

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Abstract

A liquid chromatography-tandem mass spectrometric (LC–MS–MS) method for the highly sensitive determination of a new bone-anabolic agent, TAK-778 in human serum was developed. The internal standard (I.S.) used was deuterated TAK-778. TAK-778 and I.S. were extracted from serum samples with diethyl ether at neutral pH. A turbo ion spray interface was used as the ion source of LC–MS–MS, and the analysis was performed in the selected reaction monitoring mode. The lower limit of quantification was 0.02 ng/ml when 0.4 ml of serum was used, and the standard curve was linear in the range of 0.02–10 ng/ml. The method was precise; the intra- and inter-day precision of the method was not more than 17.9%. The accuracy of the method was good with the deviations between added and calculated concentration of TAK-778 being typically within 9.0%. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: TAK-778; Bone-anabolic agent; Quantification; LC–MS–MS

1. Introduction

TAK-778 (Fig. 1), (2*R*,4*S*)-(-)-*N*-(4-dimethoxyphosphorylmethylphenyl)-1,2,4,5-tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxyamide, is a novel osteoblast differentiation promoting compound [1]. TAK-778 at a concentration of 10^{-6} M promoted potently bone-like nodule formulation in rat bone marrow stromal cell culture. Furthermore, the new bone area of bony defect rats given 4 weeks sustained-release (SR) microcapsule of TAK-778 (TAK-778-SR) was increased dose-dependently [2–5].

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According to these characteristics, TAK-778 is currently under development as a new bone-anabolic agent using SR formulation.

In order to proceed the clinical trials of TAK-778-SR, an analytical method was required to determine the concentration of TAK-778 in serum of humans administrated TAK-778-SR intramuscularly. An ELISA method for quantifying TAK-778 with the limit of quantification of 0.5 ng/ml using 200 μl of rat serum was developed and reported [6]. Although this method would be applied to the quantification of TAK-778 in human serum, several steps including preparation of anti-TAK-778 antibodies, preparation of wells coated with anti-TAK-778 antibodies, and reaction of TAK-778 may lead to delay the quantification process in the clinical trials. In addition, the lower limit of quantification in the ELISA method was not sensitive enough to measure possibly lower levels of TAK-778 in human serum after administration of TAK-778-SR. Therefore, a more sensitive and rapid method for quantifying TAK-778 in serum of humans was required to characterize the pharmacokinetics properties of TAK-778 in human and allow the clinical trials to proceed efficiently.

This paper describes the development of a highly sensitive LC–MS–MS method for the determination of TAK-778 in human serum.

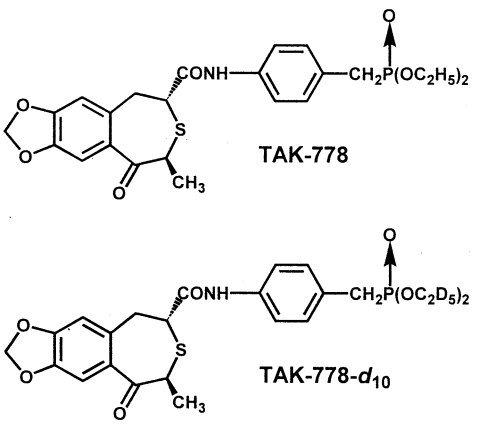

Fig. 1. Chemical structures of TAK-778 and TAK-778-d_{10}.

2. Experimental

2.1. Chemicals

An authentic sample of TAK-778 (Fig. 1) was synthesized in the Pharmaceutical Production Division, Takeda Chemical Industries (Osaka, Japan). The chemical purity for the TAK-778 was 99.8%. Deuterium-labeled TAK-778 (TAK-778-d_{10}; Fig. 1), the internal standard (I.S.) for TAK-778 was prepared in the Pharmaceutical Research Division, Takeda Chemical Industries. The isotope purity for the TAK-778-d_{10} was 99.9%. HPLC grade methanol and analytical grade diethyl ether, acetic acid, disodium hydrogen phosphate, and potassium dihydrogen phosphate were purchased from Wako Pure Chemicals (Osaka, Japan). Drug free human serum was obtained from healthy volunteers.

2.2. Instrumentation and condition for LC–MS–MS analysis

The LC–MS–MS system consisted of a Waters Alliance 2690 separation system module attached with in-line degasser, sample cooler, column heater, seal wash (Waters, MA, USA) and API365 with turbo ion spray interface (PE Sciex, ON, Canada). The HPLC column used was a YMC J'sphere ODS-M80 (150×2.0 mm I.D., YMC, Kyoto, Japan). A mixture of water–methanol–acetic acid (15:85:0.2, v/v/v) was used as the mobile phase at a flow-rate of 0.2 ml/min. The column temperature was 40 °C. MS-MS detection was performed by the selected reaction monitoring (SRM) from the protonated molecule ($[M + H]^+$) of the analyte to its product ion under the positive ion mode. The product ion efficiency of the $[M + H]^+$ was optimized by varying the turbo ion spray, orifice and focusing ring voltages using an auto-tune program (LC2 tune; PE Sciex). The flow rates of the curtain gas (N_2; 99.999% purity), neblizer gas (zero grade air; 99.999% purity), and heater gas (zero grade air; 99.999% purity) were 1.25, 1.31, and 7 l/min, respectively. The turbo probe temperature was 425 °C. The collision-induced dissociation (CID) of the analytes was performed at quadrupole 2 using N_2 gas

at a pressure of 2.8×10^{-5} Torr (1 Torr = 133.322 Pa) and collision energy was set at -25 eV. The monitoring ions (precursor ion → product ion) for SRM analysis were set to m/z 506 → 216 for TAK-778 and m/z 516 → 222 for TAK-778-d_{10}. The scan dwell time and multiplier voltage were set to 2 s and 2.0 kV, respectively.

2.3. Preparation of the standard solutions

Each stock solution of TAK-778 and I.S. (each 100 μg/ml) was prepared by dissolving about 2 mg of each compound into 20 ml of methanol. The stock solution of TAK-778 was serially diluted with methanol to provide working solutions with concentrations of 0.04, 0.1, 0.2, 1, 2, 10, and 20 ng/ml. In addition, the stock solution of TAK-778-d_{10} was serially diluted with methanol to prepare the working I.S. solution with concentration of 1 ng/ml.

2.4. Preparation of spiked standards and quality control samples

The working solution (200 μL) was evaporated to dryness under a stream of nitrogen gas at 40 °C and the residues were dissolved in 400 μl of drug-free human serum to prepare standard samples with concentrations of 0.02, 0.05, 0.1, 0.5, 1, 5, and 10 ng/ml. Similarly, QC samples with the three concentrations of 8 (high), 1 (medium), and 0.1 ng/ml (low) were prepared from the stock and working solutions described above. These samples were stored at -80 °C.

2.5. Extraction procedure

A 0.4 ml aliquot of serum sample was mixed with 0.4 ml of a mixture of disodium hydrogen phosphate (50 mM)-potassium dihydrogen phosphate (50 mM) (pH 7.0) and 0.2 ml of the working I.S. solution. TAK-778 and I.S. were extracted with diethyl ether (5.0 ml) using a shaker for 5 min. The organic layer was evaporated to dryness, and the residue was reconstituted in 0.2 ml of a mixture of water–methanol–acetic acid (50:50:0.2, v/v/v). The 100 μl portion of the solution was injected into LC–MS–MS system.

2.6. Calculation and validation

The standard curve was obtained by a $1/C$ weighted least-squares linear regression on the peak area ratio of each analytes to I.S. versus the concentrations of each analyte in the spiked samples:

$$y = a + b \times C_{\text{theor}}$$

where, y, C_{theor}, a and b are the peak area ration, the analyte concentration, the y-intercept, and the slope, respectively. The concentration (C_{obs}) was calculated from the equation of the standard curve:

$$C_{\text{obs}} = (y - a)/b$$

The intra-assay precision and accuracy were determined by analyzing five sets of the spiked standard samples with concentrations of 0.02, 0.05, 0.1, 0.5, 1, 5, and 10 ng/ml. The intra-assay validation was performed by analyzing QC samples in five replicates on three separate days.

2.7. Stability

The stabilities of TAK-778 in human serum were evaluated as the residual ratio. The residual ratio was calculated by comparing the peak area ratio (TAK-778 vs. I.S.) of TAK-778 extracted from stored samples to that from freshly prepared samples.

2.8. Recovery

The extraction recovery of analyte from human serum was calculated by comparing the peak area ratio (TAK-778 vs. I.S.) of extracted TAK-778 to that of non-extracted TAK-778. Extracted TAK-778 was prepared by the extraction procedure described above. For the non-extracted sample, TAK-778 was added to the drug-free serum extract prepared by the same extraction procedure at the same final concentration as the extracted TAK-778. The recovery of the I.S. was determined by the same procedure as described above except for reversal of the spiking order of the I.S. and TAK-778.

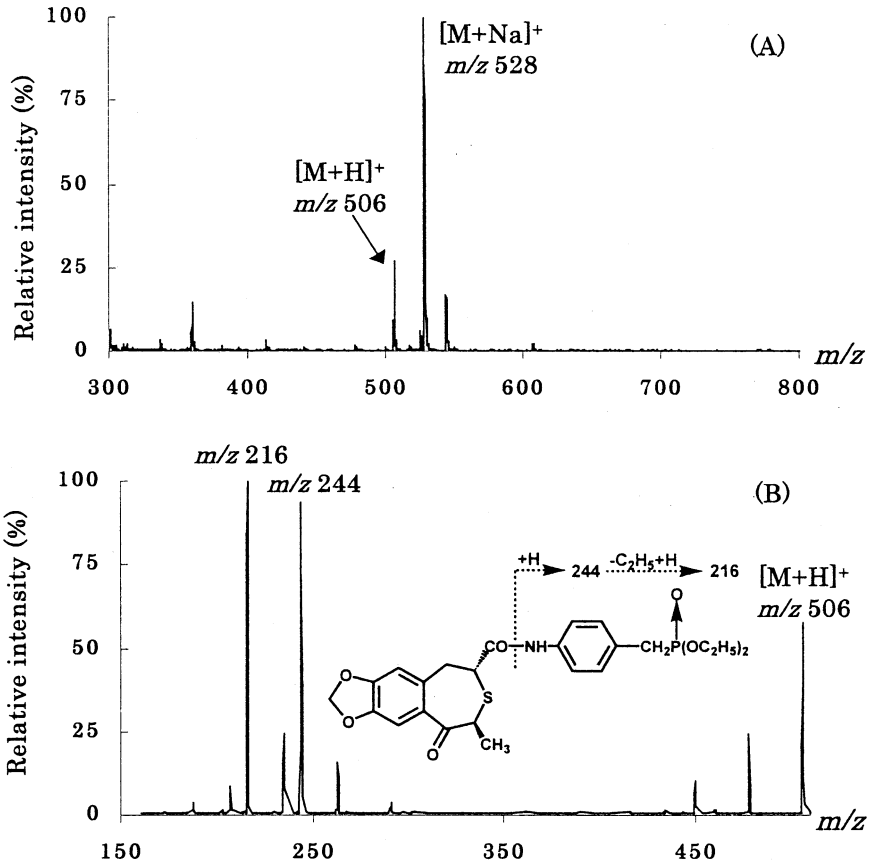

Fig. 2. Full ion mass spectrum (A) and product ion mass spectrum (B) of TAK-778.

3. Result and discussion

3.1. Mass spectrometry

The full ion mass spectrum of TAK-778 gave the $[M + H]^+$ and sodium ion adduct molecule ($[M + Na]^+$) appearing at m/z 506 and 528, respectively (Fig. 2A). The base peak in the full ion mass spectrum was $[M + Na]^+$, however, the intensity of $[M + Na]^+$ is likely to be influenced by sodium concentration which is difficult to control [7]. Therefore, $[M + H]^+$ was selected as precursor ion for analysis. In the product ion mass spectrum of the $[M + H]^+$ of TAK-778, two characteristic fragment ions were detected at m/z 244 and 216. The fragment ions at m/z 244 and 216 were formed by the cleavage of the amide bond of TAK-778 and the loss of ethyl moiety from m/z 244, respectively (Fig. 2B). As the result of autotuning with the LC2 Tune program, the base ion peak in the product ion mass spectrum was observed at m/z 216. On the basis of these observations, the monitoring ions on the SRM analysis were set to m/z $506 \rightarrow 216$.

In the full ion mass spectrum of TAK-778-d_{10}, $[M + H]^+$ and $[M + Na]^+$ were detected at m/z 516 and 538, respectively. Furthermore, the base product ion peak at m/z 222, which was formed by the same manner to non-deuterated TAK-778, was given by product ion scanning of $[M + H]^+$ of TAK-778-d_{10}. Therefore, the monitoring ions for TAK-778-d_{10} were set to m/z $516 \rightarrow 222$.

3.2. Chromatography

For the HPLC column, the reversed-phase column was selected for the analysis according to the hydrophobic properties of analyte. When TAK-778 was injected into the reversed-phase HPLC column with a mobile phase of water–methanol in a preliminary test, TAK-778 eluted as broad-tailing peaks from the column. Hence, acetic acid was added to the mobile phase to obtain sharp and symmetrical elution peak of TAK-778. The optimization of mixture ratio of water, methanol, and acetic acid was performed under SRM conditions by monitoring the peak intensity, peak shape, and retention time of TAK-778. As the result, a mixture ratio of water, methanol, and acetic acid (15:85:0.2, v/v/v) was found to be appropriate for both sensitivity and peak shape.

Fig. 3 shows typical SRM chromatograms of human serum samples prepared by the method described in Section 2. The retention times of TAK-778 and TAK-778-d_{10} were both 3.4 min and the chromatograms of drug-free human serum were free of endogenous peaks at the retention times of TAK-778 and TAK-778-d_{10}.

3.3. Linearity, precision, stability, recovery, and lower limit of quantification

The standard curves were obtained by the analysis of standard solutions over the sample concentration range from 0.02 to 10 ng/ml. The least-squares regression fit showed good linearity with correlation coefficients higher than 0.999. The linearity of the standard curves for TAK-778 added to human serum was reproducible as indicated by the coefficients of variation (C.V.s) in the slope of the different standard curves.

The intra- and inter-day precision and accuracy data of the analytical procedure are presented in

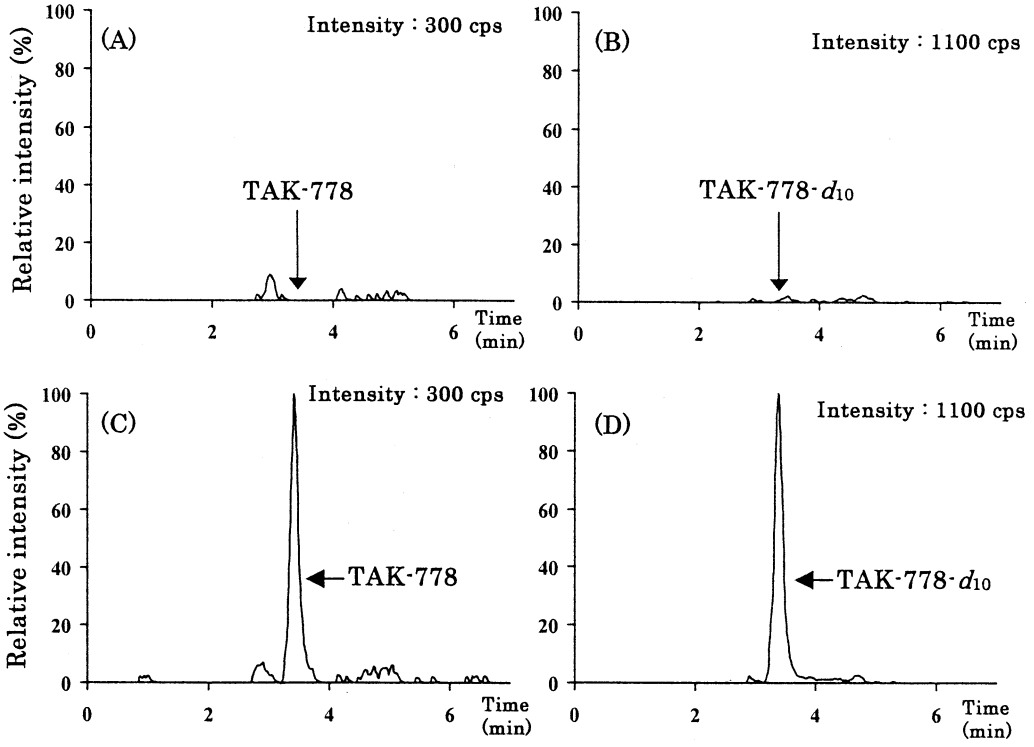

Fig. 3. Representative SRM chromatograms of extract from drug-free serum (A,B) and from serum spiked with TAK-778 (0.02 ng/ml) and I.S. (0.5 ng/ml) (C,D). Detection mode for TAK-778 (m/z 506 → 216) (A,C) and for TAK-778-d_{10} (m/z 516 → 222) (B,D). Figures in parentheses indicate m/z values of precursor ion and product ion, respectively.

Table 1
Precision and accuracy of the method

Nominal concentration (ng/ml)	Intra-day			Inter-day		
	Found concentration (ng/ml)	C.V. (%)	R.E. (%)	Found concentration (ng/ml)	C.V. (%)	R.E. (%)
0.0200	0.0218	17.9	9.0	–	–	–
0.0500	0.0488	8.2	−2.4	–	–	–
0.100	0.0945	4.4	−5.5	0.0994	2.8	−0.6
0.500	0.488	4.5	−2.4	–	–	–
1.00	1.01	3.0	1.0	1.02	2.9	2.0
5.00	5.00	2.0	0.0	–	–	–
8.00	–	–	–	8.11	1.6	1.4
10.0	10.0	3.0	0.0	–	–	–

Mean value of five determinations. –, not examined.

Table 1. The precision and accuracy were evaluated by the C.V. and relative error (R.E.), respectively. The intra-day C.V. and R.E. were calculated from five observed concentrations at each spiked standard concentration, and those for the inter-day were determined by the mean observed QC concentration ($n = 5$) on 3 separate days. The intra-day C.V. and R.E. of analytes were not more than 17.9 and 9.0%, respectively. The inter-day variability indicated that the C.V. and R.E. for the analytes were 2.9 and 2.0%, respectively.

The residual ratios of TAK-778 in human serum stored for 2 h at room temperature and for 1 month at −80 °C were 96.9–98.6 and 90.1–91.3%, respectively.

The extraction recoveries of TAK-778 and TAK-778-d_{10} from human serum were 86.1–93.5 and 112.2%, respectively (Table 2).

From these results, it was clear that the method was reliable within that range and that the lower limit of quantification was 0.02 ng/ml in human serum.

3.4. Application

The LC–MS–MS method was applied to the quantification of TAK-778 in serum samples from human subjects from a clinical study after intramuscular administration of TAK-778-SR. A typical profile of TAK-778 for up to 552 h after intramuscular administration of TAK-778-SR (5 mg as TAK-778) is shown in Fig. 4. Even 552 h

Table 2
Recoveries of TAK-778 and TAK-778-d_{10} from human serum

Concentration (ng/ml)	Recovery (%)	
	TAK-778	TAK-778-d_{10}
0.1	93.5	–
0.5	–	112.2
1	86.1	–
10	93.3	–

Mean values ($N = 2$). –, not examined.

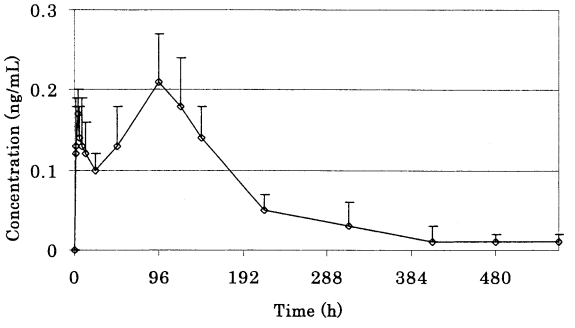

Fig. 4. Concentration of TAK-778 in serum of humans administered TAK-778-SR (5 mg as TAK-778) intramuscularly. Mean values ($N = 6$).

after dosing, TAK-778 could be quantified using this method.

4. Conclusion

This paper describes the development of a highly sensitive and simple LC–MS–MS method with liquid–liquid extraction for determination of TAK-778 in human serum. The method established can quantify TAK-778 with a lower quantification limit of 0.02 ng/ml using 0.4 ml of serum, and proved to be fast and reproducible with an analysis time of 7 min for one sample. It is capable of use in elucidating the pharmacokinetic profiles of TAK-778 in human given TAK-778-SR.

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