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Analysis of Risedronate and Related Substances by Ion-Pair Reversed-Phase High-Performance Liquid Chromatography with Evaporative Light-Scattering Detection

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A simple method has been developed for the analysis of risedronate and related substances by ion-pair reversed-phase high-performance liquid chromatography (RPLC) with evaporative light-scattering detection (ELSD). After optimization of the chromatographic conditions, satisfactory separation of the compounds was achieved on an Intersil C_8 column with an isocratic mobile phase: 8:4:88 (v/v) acetonitrile–methanol–12 mM ammonium acetate buffer containing 35 mM *n*-amylamine (pH 7.0). The mobile-phase flow rate was 1.0 mL min^{-1}. The calibration plot was linear in the range of 352 to 1760 μg mL^{-1} for risedronate. The precision and reproducibility were 0.3 and 0.5%, respectively. The average recovery of risedronate was 100.4% and the RSD was 0.6%. The method was validated and shown to be precise, accurate, and specific for the assay of risedronate in both bulk material and dosage forms. The proposed liquid-chromatographic method can be satisfactorily used for the quality control of risedronate.

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Introduction

Risedronate monosodium ([1-hydroxy-2-(3-pyridine)ethylidene]-bisphosphonic acid monosodium salt; Fig. 1) belongs to a group of chemical compounds known as bisphosphonates. This new third-generation bisphosphonate has been used to treat patients with bone disease, for example Paget's disease, malignant hypercalcemia, and postmenopausal osteoporosis.[1,2] During the production, storage, and clinical use of risedronate, related substances, such as phosphite, 2-(3-pyridine)acetic acid, and phosphate may be present and may cause adverse effects. It is necessary to establish a quality-control method for an easy simultaneous assay of risedronate and related substances.

Many liquid chromatographic methods have been reported for the analysis of bisphosphonates. Because the compounds have more than one ionizable group, they are hard to retain on non-polar stationary phases, for example C_8 or C_{18}; it is thus necessary to use an ion-exchange chromatography or add an ion-pairing reagent to the mobile phase.[3–11] The development of chromatographic methods for this class of compound is also challenging, owing to the lack of a chromophore for conventional UV or fluorescence detection. HPLC measurements of bisphosphonates have been accomplished by introducing a chromophore, a fluorophore, or an electrochemically active group into the molecules by pre- and postcolumn derivatization, thus enabling UV,[4,5] fluorescence,[12,13] or electrochemical detection,[14] respectively. Other direct detection methods without derivatization have included on-line flame photometric detection,[15] conductivity detection,[16] electrochemical detection,[17] and mass spectrometry.[7]

Because there is no chromophore for UV or fluorescence detection, and no suitable groups for derivatization, few analytical methods had been described for risedronate. A method based on ion-exchanged chromatography with UV detection has been reported for the analysis of risedronate.[18] In that work, copper ion was added into the mobile phase to form a complex with risedronate, and the complex was detected at 240 nm. This system could not be used to determine phosphite, 2-(3-pyridine)acetic acid, and phosphate, however, which are regarded as being risedronate-related substances. Chromatographic detection of these three compounds is also complicated because of the physical properties exploited by, *e.g.*, UV and amperometric detectors. Consequently, it is difficult to find a chromatographic system for the four compounds (risedronate and related substances) that have sufficiently high stability, efficiency, and resolution for drug formulation analysis.

We have developed an ion-pair reversed-phase high-performance liquid chromatographic (RPLC) method with evaporative light-scattering detection (ELSD) for the assay of risedronate and related substances in bulk material and pharmaceutical dosage forms. The nonvolatile mobile phase usually used in RPLC comprising phosphate buffer and tetraalkylammonium was successfully replaced by a volatile

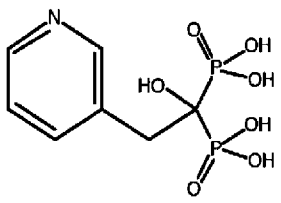

Fig. 1 Chemical structure of risedronate.

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system containing ammonium acetate buffer and *n*-amylamine, which is suitable for ELSD. Satisfactory separation of risedronate and related substances was achieved by isocratic elution. This method was performed with commercially available, conventional HPLC without the need to control the column temperature. We therefore fulfilled the objective of this work to develop a convenient, readily available, and reliable RPLC-ELSD method for use in the quality-control of risedronate and related substances during production or storage.

Experimental

Reagents and chemicals

Purified risedronate, 2-(3-pyridine)acetic acid, three batches of risedronate injection and three batches of bulk material were provided by the Pharmaceutical Factory of Hebei Xingtai (Xingtai, China).

Acetonitrile, HPLC grade, was purchased from Dima Technology (USA). Methanol, HPLC grade, was purchased from Tedia (USA); *n*-amylamine was purchased from Acros Organics (NJ); ammonium acetate, phosphite, and potassium dihydrogen phosphate were analytical grade. Double-distilled water was used for mobile-phase preparation.

Equipment and chromatographic conditions

HPLC was performed with an LC-10At precision pump (Shimadzu Instruments, Japan), a Rheodyne 7725 injector with 20-μL loop, and a Tj-912 chromatography workstation (Software Company of Tianjin Xietong, China). Risedronate and related substances were separated on an Intersil C_8 column (150 mm × 4.6 mm i.d., 5 μm particles) (GC Science, Japan). The mobile phase was a mixture of acetonitrile (8%), methanol (4%), and 12 mM ammonium acetate buffer (88%) containing 35 mM *n*-amylamine, adjusted to pH 7.0 with acetic acid. The flow-rate was 1.0 mL min^{-1}. The injection volume was 20 μL.

The ELSD was an Alltech (USA) 2000. High-purity nitrogen was used as a nebulizer gas at a flow-rate of 2.7 L min^{-1}. The impactor position was set at off. The drift-tube temperature was 105°C. The gain was set at 4.

Preparation of solutions

Stock standard solutions of risedronate and related substances (phosphite, 2-(3-pyridine)acetic acid, and phosphate) were prepared in water. Each solution was further diluted to give a series of working standards. They were 176 μg mL^{-1} for risedronate, 3 μg mL^{-1} for phosphite, 10 μg mL^{-1} for 2-(3-pyridine)acetic acid, and 5 μg mL^{-1} for phosphate. A solution modeling a dosage form of risedronate was prepared according to the commercial formulation of risedronate injection reported by Boehringer Mannheim (Germany). Each vial contained 1.0 mg risedronate, 8.6 mg sodium chloride, and 0.2 mg sodium acetate in 1.0 mL injection water. The solutions used for determination of the recovery of risedronate contained the compound at 80, 100, and 120% of the level in risedronate injection. For the assay, three batches of risedronate bulk material were dissolved in water to furnish test solutions containing 1000 μg mL^{-1} risedronate. Three batches of risedronate injection (1.0 mg mL^{-1}) were directly used for the assay.

In an attempt to study the method specificity, forced degradation of risedronate samples was conducted. Risedronate in aqueous solution was irradiated with ultraviolet light. Oxidative degradation was attempted by adding 3% hydrogen peroxide.

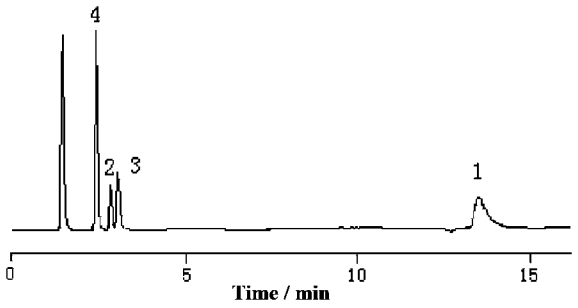

Fig. 2 Typical chromatogram obtained from risedronate and related substances on a C_8 column with 8:4:88 (v/v) acetonitrile–methanol–12 mM ammonium acetate buffer containing 35 mM *n*-amylamine, pH 7.0, as the mobile phase. Peaks: 1, risedronate; 2, phosphate; 3, phosphite; 4, 2-(3-pyridine)acetic acid.

Results and Discussion

The primary objective of this work was to develop a simple and reliable chromatographic method for the assay of risedronate with sufficient separation between the main component, residues from the synthesis, for example 2-(3-pyridine)acetic acid and phosphite, and possible impurities resulting from oxidation/decomposition, for example phosphate.

Optimization of chromatographic conditions

The initial work involved the optimization of chromatographic conditions on the basis of satisfactory separation of 2-(3-pyridine)-acetic acid, phosphite, and phosphate both from each other and from risedronate, and with a reasonable retention time and good peak shape for risedronate. The ELSD conditions were optimized to enhance the sensitivity of detecting of risedronate.

Chromatographic column Intersil C_8 and C_{18} columns were evaluated as a stationary phase for the assay. With the C_{18} column, when the risedronate retention was reasonable ($k < 10$), peaks of the related substances were unresolved from each other. Attempts to resolve risedronate-related substances completely by varying the pH, buffer concentration, ion-pairing reagent concentration, and organic modifier, were unsuccessful. The replacement of the C_{18} column with a C_8 column resulted in a satisfactory separation of risedronate and related substances under the optimized chromatographic conditions (Fig. 2), so the C_8 column was adopted for the analysis.

Effect of concentration of ion-pairing reagent. Because risedronate and its related substances are strongly polar and ionic, RPLC was regarded as being a useful technique for separating of the compounds. Different tetraalkylammonium salts are the ion-pairing reagents most commonly selected in the RP-HPLC separation of bisphosphonate.[10,11,17] Volatile ion-pairing reagents, such as *n*-butylamine, *n*-amylamine, *n*-hexylamine, and *n*-octylamine, were also evaluated to achieve a suitable retention of risedronate and related substances. The volatile ion-pairing reagent *n*-amylamine was selected because it enabled reasonable retention of the compounds and, because of its low boiling point, was likely to enhance the sensitivity of ELSD.

The effect of the *n*-amylamine concentration on the retention of risedronate and related substances at constant concentrations of buffer (12 mM, pH 7.0) and organic modifier (acetonitrile 8%, methanol 4%) was studied. The retention of risedronate and related substances increased with increasing *n*-amylamine concentration from 0 to 70 mM. The optimum concentration

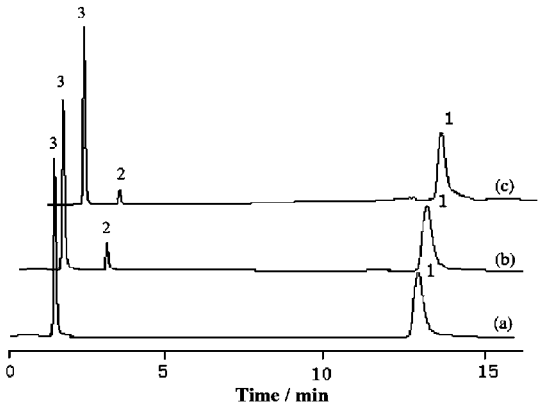

Fig. 3 Chromatograms obtained from risedronate treated with (a) water, (b) ultraviolet light, and (c) H_2O_2. The chromatographic conditions were similar to those used for Fig. 2. Peaks: 1, risedronate; 2, phosphite; 3, 2-(3-pyridine)acetic acid.

was 35 mM, because it enabled adequate separation in a reasonable time.

Effect of the mobile-phase composition. The analysis of risedronate and related substances was investigated using mobile phases of different composition. Acetonitrile was chosen as an organic modifier, because it improved the peak shape of risedronate substantially. The retention times of the analytes decreased as the concentration of the organic modifier was increased. Satisfactory separation of the four compounds was achieved when the acetonitrile concentration was 8%, but the retention time of risedronate was too long (>25 min), so a small amount of methanol (4%) was added to the mobile phase. This enabled satisfactory separation of the four compounds in a reasonable time.

Effect of buffer concentration. The effect of the buffer concentration (5 – 50 mM) was studied at a constant *n*-amylamine concentration (35 mM), pH 7.0, and organic modifier concentration (acetonitrile 8%, methanol 4%). Increasing the buffer concentration reduced the tailing factor of risedronate, and reduced the compound retention times. The system noise increased with increasing buffer concentration, as reported by Dreux *et al*.[19] A concentration of 12 mM was selected for further study, because it resulted in the best compromise between the peak shape, resolution, and low system noise.

Effect of mobile phase pH. Risedronate is strongly acid compared with 2-(3-pyridine)acetic acid, phosphite, and phosphate, so it is important to select the pH that enables us a suitable retention of risedronate and related substances with adequate separation from each other. The effect of a pH on the retention time of each analyte was investigated over the pH range 4.0 – 7.2. This showed that the retention of risedronate and related substances increased with increasing mobile phase pH. At pH 7.0, the separation of 2-(3-pyridine)acetic acid, phosphite, and phosphate was satisfactory. This revealed that only under higher pH conditions were these three compounds present as anions, and thus able to form neutral ion pairs with the *n*-amylamine positive ion, and be retained on the C_8 column. The choice of pH 7.0 for the mobile phase enabled not only excellent separation and reasonable retention time, but also resulted in a long column life.

Optimization of the ELSD. The two basic conditions that determine the ELSD response are the nebulizer gas flow-rate and the drift-tube temperature. The optimum conditions were investigated by observing the intensity of the risedronate signal at different gas flow-rates and drift-tube temperatures. The optimum nebulizer gas (high-purified nitrogen) flow-rate in this work was found to be 2.7 mL min^{-1}, and the optimum drift-tube temperature was 105°C.

Table 1 Linear-regression calibration data for risedronate and related substances

Compound	Concentration range/μg mL^{-1}	Equation of the calibration plot	R^2
2-(3-Pyridine)-acetic acid	23 – 372	$Y = 2.1783x + 4.0827$	0.9982
Phosphate	92 – 460	$Y = 1.1409x + 3.9924$	0.9988
Phosphite	24 – 384	$Y = 1.7198x + 3.1431$	0.9990
Risedronate	352 – 1760	$Y = 2.3082x - 0.0358$	0.9994

Validation of the assay

The analytical method was validated by testing such properties as the specificity, linearity, precision, recovery, detection limit, and reproducibility under the optimized chromatographic conditions.

Specificity. The specificity of the method was studied for risedronate and related substances. The obtained chromatogram (Fig. 2) showed that the resolution between all compounds was adequate. The specificity of the method was further assessed by the analysis of risedronate solutions oxidized with H_2O_2 (the risedronate solutions was added into 30% H_2O_2 solutions for 30 min), or subjected to irradiation with ultraviolet light (40 W; effective irradiation height, 25 cm; irradiation for 6d). The decreased content of risedronate respectively was 11.6 and 9.2% after the degradation experiment was completed, according to a calculation. Figures 3a – 3c show the presence of another peak in addition to that of risedronate. The longer risedronate solutions were oxidized or irradiated, the more the area of the risedronate peak decreased, and that of the other peak increased. The peaks were, however, well separated, so the proposed method is suitable for a specific determination of risedronate.

Linearity. The linearity was tested by the three-fold injection of a mixture of risedronate and related substances at different concentrations: from 352 to 1760 μg mL^{-1} for risedronate, from 24 to 384 μg mL^{-1} for phosphite, from 23 to 372 μg mL^{-1} for 2-(3-pyridine)acetic acid, and from 92 to 460 μg mL^{-1} for phosphate. In ELSD, the detector response is given by $y = ax^b$, where y is the peak area, x is the sample amount and a and b are numerical coefficients. Plots of the peak area against the amount of sample in double logarithmic coordinates were linear for all compounds. The linear correlation coefficients (R^2) ranged from 0.9982 to 0.9994. Detailed results are given in Table 1.

Precision. The precision, expressed as the relative standard deviation (RSD), based on seven replicate injections of one standard solution of risedronate, was 0.3%.

Reproducibility. The reproducibility, expressed as RSD, based on injections of seven standard solutions of identical risedronate concentration, was 0.5%.

Stability. Standard and sample solutions of risedronate were assayed after 0, 1, 2, 4, 8, 12, and 24 h. The RSD values of the peak areas were 0.5 and 0.8%, respectively.

Detection limits. Detection limits, based on a signal-to-noise ratio of three, were determined for all compounds by serial dilution of standard solutions. They were 176 μg mL^{-1} for

Table 2 Recovery of risedronate

Amount added/μg mL^{-1}	Amount found/μg mL^{-1}	Recovery, %
798.6	803.5	100.6
798.6	805.3	100.8
798.6	792.3	99.2
998.3	1005.6	100.7
998.3	1008.4	101.0
998.3	1006.9	100.8
1198.0	1201.5	100.2
1198.0	1208.4	100.8
1198.0	1191.2	99.4

The average recovery was 100.4% (RSD, 0.6%).

Table 3 Comparison of the results from RPLC-ELSD and from the molybdenum blue spectrophotometric method for risedronate

Product	*N*	RPLC-ELSD		Spectrophotometry	
		Claim	Found ± SD	Claim	Found ± SD
Bulk material	5	100%	98.5 ± 0.9%	100%	99.0 ± 1.2%
Risedronate injection	5	1.0 mg mL^{-1}	0.96 ± 0.07 mg mL^{-1}	1.0 mg mL^{-1}	0.94 ± 0.10 mg mL^{-1}

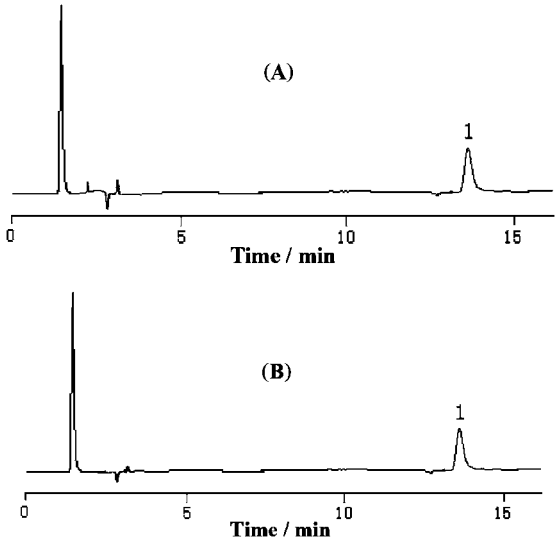

Fig. 4 Chromatograms obtained from risedronate in bulk material (A) and risedronate injection (B). Peak: 1, risedronate.

risedronate, 3 μg mL^{-1} for phosphite, 10 μg mL^{-1} for 2-(3-pyridine)acetic acid, and 5 μg mL^{-1} for phosphate.

Recovery. The solutions analyzed for determining of the recovery of risedronate were 80, 100, and 120% of the levels of risedronate in injection solution. The average recovery of risedronate was 100.4% and the RSD was 0.6% (Table 2). This indicated that sodium acetate and sodium chloride did not interfere with the determination of risedronate in the dosage form.

Analytical application

Sample detemination. Results from the assay of risedronate in bulk material and a dosage formulation are given in Table 3. The precision of the data and the agreement between the label claim and the amount found were excellent. The method was validated for risedronate and its dosage form, using the analytical criteria precision, accuracy, and linearity. Potential by-products and formulation components of the dosage form, including sodium acetate and sodium chloride, did not interfere with the assay procedure for risedronate. At least six columns from the same manufacturer were tested, and all save similar results (Figs. 4A and 4B).

Stability study. Risedronate can be decomposed by oxidization with H_2O_2 or by irradiation with ultraviolet light (Figs. 3b and 3c). The analysis of deliberately degraded samples showed that the separation of related substances from risedronate was excellent, so the method can be used to determination risedronate. The results of our study have indicated that both risedronate bulk material and commercial injection formulation have good stability under normal storage conditions.

Comparison of RPLC with the molybdenum blue spectrophotometric method. The data given in Table 3, obtained by both RPLC-ELSD and the molybdenum blue spectrophotometric method, attest to the equivalence of the methods. There was no significant difference between the results from the two methods. Because phosphorus atom-content was determined by molybdenum blue spectrophotometry, the method cannot be used for the assay of risedronate and related substances. RPLC-ELSD has the advantage of its specificity, by which risedronate can be separated from related substances. This method was convenient, accurate, and precise for the assay of risedronate.

Conclusions

RPLC-ELSD proved to be suitable for simple direct determination of risedronate. Efficient separation of risedronate and related substances was achieved on a C_8 column with commercially available, conventional HPLC equipment. A volatile mobile phase containing ammonium acetate buffer and *n*-amylamine was suitable for ELSD. The RPLC-ELSD method has been shown to be equivalent to the molybdenum blue spectrophotometric method, and has good specificity. This newly developed method also enables the direct measurement of risedronate in different dosage forms without the need for derivatization. The method sensitivity was sufficient for the analysis of risedronate in bulk material and a commercial dosage form. The method is also applicable as a simple quality-control method for risedronate during production and storage. The result has led us to investigate a wide range of applications of this methodology, especially for the quality control of other bisphosphonates and related substances.

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