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Liquid chromatography–mass spectrometry analysis of five bisphosphonates in equine urine and plasma

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ABSTRACT

Bisphosphonates are used in the management of skeletal disorder in humans and horses, with tiludronic acid being the first licensed veterinary medicine in the treatment of lameness associated with degenerative joint disease. Bisphosphonates are prohibited in horseracing according to Article 6 of the International Agreement on Breeding, Racing and Wagering (published by the International Federation of Horseracing Authorities). In order to control the use of bisphosphonates in equine sports, an effective method to detect the use of bisphosphonates is required. Bisphosphonates are difficult-to-detect drugs due to their hydrophilic properties. The complexity of equine matrices also added to their extraction difficulties. This study describes a method for the simultaneous detection of five bisphosphonates, namely alendronic acid, clodronic acid, ibandronic acid, risedronic acid and tiludronic acid, in equine urine and plasma. Bisphosphonates were first isolated from the sample matrices by solid-phase extractions, followed by methylation with trimethylsilyldiazomethane prior to liquid chromatography – tandem mass spectrometry analysis using selective reaction monitoring in the positive electrospray ionization mode. The five bisphosphonates could be detected at low ppb levels in 0.5 mL equine plasma or urine with acceptable precision, fast instrumental turnaround time, and negligible matrix interferences. The method has also been applied to the excretion study of tiludronic acid in plasma and urine collected from a horse having been administered a single dose of tiludronic acid. The applicability and effectiveness of the method was demonstrated by the successful detection and confirmation of the presence of tiludronic acid in an overseas equine urine sample. To our knowledge, this is the first reported method in the successful screening and confirmation of five amino- and non-amino bisphosphonates in equine biological samples.

1. Background

Bisphosphonates are in a class of compound characterized by the $-C(PO_3)_2$ group acting as effective bone resorption inhibitors. These substances are used extensively in the management of skeletal disorders. Bisphosphonates are categorized as non-nitrogen-containing (e.g. tiludronic acid, clodronic acid) and nitrogen containing bisphosphonates (e.g. alendronic acid, risedronic acid, ibandronic acid) and have different mechanisms of action to inhibit osteoclast-mediated bone resorption [1]. They all share a common P–C–P moiety where the two phosphate groups are covalently linked to a carbon atom. Pharmacological properties, such as pharmacokinetics and drug strength are influenced by the chemical structures of the other functional groups (Fig. 1). They have been used clinically in human medicine for the treatment of osteoporosis, Paget's disease, malignant diseases, and in hypercalcaemia to reduce skeletal morbidity associated with bone metastases. Bisphosphonates were introduced, with tiludronic acid being the first licensed in Europe in 2011, as a veterinary medicine in the treatment of equine orthopaedic conditions [2–4]. Under the rules of racing in many countries, the presence of bisphosphonates is prohibited in samples taken from racehorses. However, a lack of understanding of their pharmacokinetics in horses has prevented the proper control of their use. A sensitive analytical method is therefore essential for elucidating their elimination profile in horses.

A number of analytical methods have been reported for the detection of bisphosphonates in human plasma and serum [5–13]; human urine [5,7,10,14–16], and equine plasma [17]. The develop-

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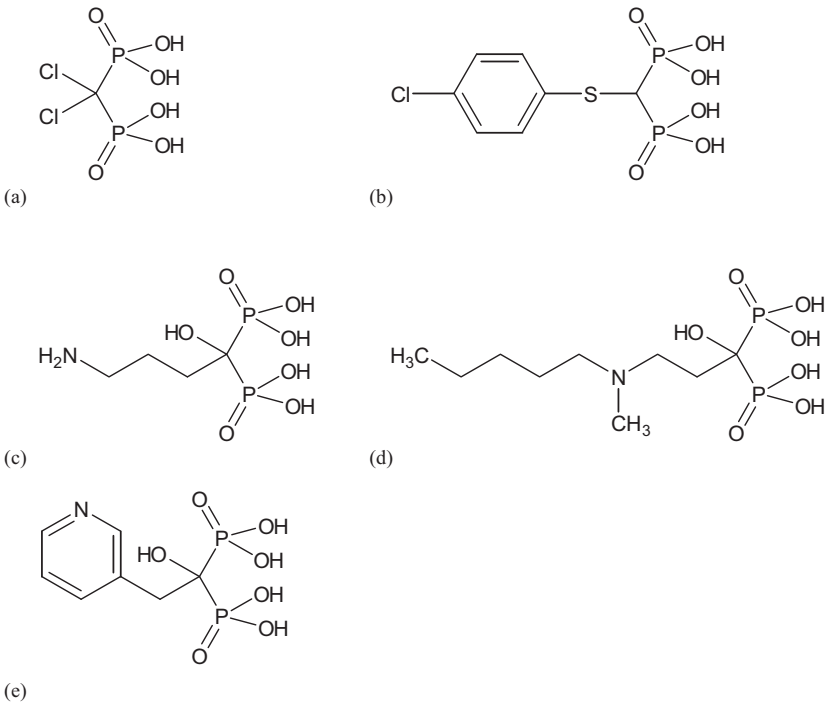

Fig. 1. Chemical structures of (a) clodronic acid, and (b) tiludronic acid in the non-nitrogen containing bisphosphonates group; and (c) alendronic acid, (d) ibandronic acid, and (e) risedronic acid in the nitrogen containing bisphosphonate group.

ment of analytical method for the screening of bisphosphonates in biological matrices is challenging due to the chemical properties of these compounds. Bisphosphonates are hydrophilic compounds and ionize extensively in aqueous solution thus making simple liquid/liquid extraction ineffective. Ion-pair extraction using solid-phase extraction with 1-octyltriethylammonium phosphate as the counter ion [15] and liquid/liquid extraction using tetrabutylammonium bromide in chloroform [18] have been reported. Another widely-used but rather tedious sample extraction procedure is to isolate bisphosphonates from human matrix by repeated co-precipitation of bisphosphonates with calcium salts under alkaline condition, followed by sample clean-up or derivatization [5,7,14,18–20]. Due to the lack of chromophores in bisphoshonates, spectrophotometric detection after chromatographic separation is usually achieved using UV or fluorescent active derivatizating reagents (alendronate with 9-fluorenylmethy chloroformate [9,14]; olpadronate with 9-fluorenylmethy chloroformate [7]; alendronate with 2,3-naphthalene dicarboxyaldehyde [5], and pamidronate with 1-naphthylisothiocyante [20]. Bisphosphonates are poorly retained in reversed phase analytical columns and one approach to providing adequate retention is by employing ion-pair chromatography with the addition of an ion-pair reagent during sample preparation. Due to the difficulties mentioned above, a wide range of analytical techniques, such as gas chromatography [21], ion chromatography [22], capillary electrophoresis–electrospray ionization mass spectrometry [23], inductively coupled plasma mass spectrometry [24], and evaporative light-scattering detection with liquid chromatography [25] have been used to try to overcome the problems in detecting bisphosphonates in human biological matrices. Simultaneous detection of different bisphosphonates is seldom reported due to the difficulty in optimizing condition for the separation and detection of bisphosphonates on the reversed phase columns.

This paper describes a liquid chromatography–mass spectrometry method which is capable of detecting five bisphosphonates in equine urine and plasma at ppb levels. To the best of our knowledge, this is the first reported method for the detection of multiple bisphosphonates in equine matrixes. This method has been successfully applied to the detection and quantification of tiludronic acid in both post-administration equine urine and blood samples.

2. Experimental

2.1. Chemicals and reagent

Reference standards of bisphosphonates are purchased, respectively, and the sources are as listed. Alendronate sodium was obtained from Apotex Inc. (Ontario, Canada). Clodronate disodium was from Schering (Berlin, Germany). Ibandronic sodium monohydrate was from Roche (New Jersey, USA). Risedronate sodium was from OSG Norwich Pharmaceuticals, Inc. (New York, USA). Tiludronic acid was from Ceva Sante Animale (Libourne, France). Ammonia solution was purchased from Merck (Darmstadt, Germany). Formic acid (>98%, RDH), methanol, trimethylsilyldiazomethane solution (2.0 M in diethyl ether) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Oasis® HLB cartridge (60 mg, 3 mL) and Oasis® WAX cartridge (60 mg, 3 mL) were purchased from Waters Corporation (Massachusetts, USA). Deionized water was generated from an in-house water purification system (Milli-Q, Molsheim, France).

2.2. Sample preparation and extraction procedures

Urine and blood samples were centrifuged at 3000 rpm for 10 min. A portion of the plasma or urine (0.5 mL) was diluted to 3 mL with deionized water and adjusted to pH 4 with hydrochloric acid. The sample was filtered through an Oasis® HLB cartridge that had been pre-conditioned with methanol (3 mL) and deionized water (3 mL). The filtrate was adjusted to pH 4 with hydrochloric acid and loaded on an Oasis® WAX cartridge that had been pre-conditioned with methanol (2 mL) and formic acid (formic acid in deionized water, pH 4; 2 mL). The cartridge was then washed with formic