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Highly sensitive fast determination of entecavir in rat urine by means of hydrophilic interaction chromatography–ultra-high-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Entecavir is a deoxyguanosine nucleotide antiviral agent with the activity against hepatitis B virus (HBV). The agent possesses a polar structure, which is predetermined for hydrophilic interaction chromatography (HILIC). Novel, fast and sensitive HILIC–UHPLC method developed in this study included separation from matrix component on BEH Amide stationary phase by isocratic elution using binary mobile phase composed of acetonitrile/5 mM ammonium acetate pH 4.0 (75:25) at flow-rate 0.3 ml/min. Analysis under RP-UHPLC conditions was also possible on BEH C18 stationary phase with mostly aqueous binary mobile phase composed of (4:96) acetonitrile/0.01% formic acid. The comparison of sensitivity of the two UHPLC–MS/MS methods both using selected reaction monitoring (SRM) for quantitation revealed only slightly higher sensitivity for HILIC determination, however much better method linearity, repeatability and accuracy. HILIC separation mode provided also more convenient conditions for straightforward coupling with solid phase extraction (SPE). Entecavir was extracted on Oasis HLB cartridge (1 ml, 30 mg) and eluted by 75% acetonitrile in water, which is actually the HILIC mobile phase used in this study. Therefore the evaporation/reconstitution step was omitted, which substantially accelerated the sample preparation step. The method was validated using stable isotopically labeled internal standard entecavir-$C_2{}^{13}N^{15}$, which is the most appropriate internal standard. Validation results demonstrated good method accuracy (with < 5% error, and 26% at LOQ), recovery (87–114%), precision (<4% RSD), selectivity and sensitivity (LOQ = 100 pg/ml). The matrix effects determined by both post-column infusion method as well as post-extraction addition method were negligible (<15%).

1. Introduction

Entecavir is a novel antiviral agent used in the treatment of hepatitis B virus (HBV). It is approved for the therapy of chronic hepatitis B similarly as five other drugs including interferon α-2a, lamivudine, telbivudine, pegylated interferon α-2a and adefovir dipivoxil [1]. Entecavir (2-amino-9-[(1-S, 3R, 4S)-4-hydroxy-3(hydroxymethyl)-2-methylindenecyclopentyl]-3H-purin-6-one) is a guanine nucleoside analogue, that inhibits HBV DNA polymerase at both the priming and elongation steps required for viral replication [2,3].

Entecavir, as a small (MW = 277.12) and polar molecule ($\log P = -1.110$), represents a significant challenge in bio-analytical method development. Due to its properties entecavir is not well-suited for conventional reversed-phase (RP) HPLC analysis and will only be retained on the column and resolved from matrix interferences at increased aqueous phase volume ratio. Such mostly aqueous mobile phases are however known to reduce the sensitivity of mass spectrometry (MS) detection. In spite of this fact, recently developed bio-analytical methods for determination of entecavir in human plasma have all used RP-HPLC [4–6]. All of them also applied LC–MS/MS using different approaches and various sample preparation steps, mostly solid phase extraction (SPE) or salting-out liquid–liquid extraction (SALLE). In spite of using RP separation mode, reported method sensitivity was at low pg/ml level due to sample pre-concentration and high injection volumes, which however leaded to serious matrix effects.

On the other hand, hydrophilic interaction liquid chromatography (HILIC) is an interesting tool for the analysis of polar compounds difficult to be retained in conventional RP systems. It provides completely different selectivity, good peak shape, high retention of polar compounds and additionally higher sensitivity in LC–MS detection due to enhanced ionization process, which is induced by mostly organic mobile phases used in HILIC [7,8].

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The most challenging issue of reliable bio-analytical method is however its accuracy and precision, which might be negatively influenced by the presence of matrix effects. Their determination recently became an important part of bio-analytical method validation [9]. There are many approaches that enable to reduce or compensate matrix effects. Among the most important, selective sample preparation approaches, efficient chromatographic separation and quantitation using stable isotopically labeled internal standards (SIL-IS) should be mentioned [10,11]. The latter is unfortunately often omitted even in newly developed LC–MS assays probably due to high cost of these standards and sometimes difficult availability [4–6]. Nevertheless, the choice of appropriate internal standard was pointed out to be crucial in order to obtain reliable results with different batches of biological materials, even in those studies using deuterium labeled SIL-IS that are supposed to compensate for any method variance. In several studies it was demonstrated, that under certain conditions high level of matrix suppression affected the ionization of analyte and its isotopically labeled analogue differently due to slight difference in retention time between the analyte and SIL-IS [12–15]. This phenomenon is supposed to be induced by deuterium isotope effect due to a small change in lipophilicity when exchanging hydrogen atom for deuterium. In all reported cases matrix effect seriously affected method accuracy and precision. For this reason, ^{13}C-, ^{15}N- or ^{17}O-labeled compounds are considered to be more appropriate than deuterium labeled internal standards. In most cases internal standards that are only structural analogues might become inappropriate. The main requirement for appropriate LC–MS internal standard is its co-elution with the analyte in order to assure stable analyte/internal standard peak area ratio and thus sufficient method accuracy and precision [10,11].

The aim of this work was to develop simple, sensitive, rapid and reliable analytical approach for the determination of entecavir by means of UHPLC–MS/MS with the aim to reduce matrix effect influence. In order to reach the goal a combination of following approaches was employed: (1) extraction and clean-up of sample by SPE; (2) dilution of sample during sample preparation step; (3) efficient UHPLC separation due to sub-2-microne particles; (4) separation selectivity and enhanced ionization due to HILIC conditions; (5) MS/MS detection using SRM experiment and (6) quantitation using SIL-IS with C^{13} and N^{15} labeling. The biological material originated from experiments with perfused rat kidney aimed to study renal excretion mechanisms of entecavir.

2. Experimental

2.1. Chemicals and reagents

Reference standard of entecavir was obtained from Santa Cruz Biotechnology, USA. Reference standard of entecavir-$C_2{}^{13}$ N^{15} was provided by Toronto Research Chemicals, Canada. Formic acid (98–100%, LC–MS grade) was obtained from Merck. Acetic acid (99.9%, LC–MS grade, Fluka), ammonium hydroxide (>25%, LC–MS grade, Fluka) and acetonitrile LC–MS grade were provided by Sigma Aldrich. Ultra-pure water was obtained with a Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and met the requirements of the European Pharmacopoeia.

2.2. Ultra high performance liquid chromatography tandem mass spectrometry

The LC–MS system consisted of UHPLC Acquity UPLC system (Waters, Prague, Czech Republic) and Quattro Micro triple quadrupole mass spectrometer (Waters, Prague, Czech Republic). The UHPLC system consisted of an ACQ-binary solvent manager, an ACQ-sample manager and an ACQ column thermostat, where the analytical column was kept at 30 °C. All injected solutions were stored in the auto-sampler at 4 °C. The partial loop with needle overfill mode was set up to inject 2 μl (using 5 μl loop). Acetonitrile was used as a strong wash (200 μl), and 20% acetonitrile in water (800 μl) was used as a weak wash solvent in RP mode. In HILIC mode 80% of acetonitrile in water (600 μl) was used as a weak wash while 100% acetonitrile was a strong wash solvent (200 μl). Under HILIC conditions entecavir was retained on Acquity BEH Amide (100 mm × 2.1 mm, 1.7 μm) analytical column using isocratic elution by mobile phase consisting of acetonitrile and 5 mM ammonium acetate pH 4.0 (75:25) at flow-rate 0.3 ml/min. Under RP conditions entecavir was retained on Acquity BEH C18 (100 mm × 2.1 mm, 1.7 μm) analytical column using isocratic elution by mobile phase consisting of acetonitrile and 0.01% formic acid (4:96) at flow-rate 0.3 ml/min.

An MS/MS triple quadrupole system Quattro Micro (Micromass, Manchester, GB) was equipped with a Multiple-Mode Ionisation Source (ESCI). The conditions were finely tuned in both HILIC and RP separation mode. An ion source in HILIC method was set-up in ESI positive ion mode as follows: capillary voltage: 3500 V, ion source temperature: 130 °C, cone voltage: 25 V, extractor: 3.0 V and RF lens: 0.3 V. The desolvation gas was nitrogen at a flow of 600 l/h and at temperature of 450 °C. Nitrogen was also used as a cone gas (150 l/h) to prevent contamination of the sample cone. The conditions for RP method were similar, except for capillary voltage, which required much lower value of 500 V. The triple quadrupole was set up to the SRM (selected reaction monitoring) experiment monitoring a quantifier transition of 277.9 > 152.2 (collision energy 15 eV) and a qualifier transition of 277.9 > 135.2 (collision energy 35 eV) for entecavir. For the internal standard entecavir-$C_2{}^{13}$ N^{15} the transition of 280.9 > 155.2 was monitored (collision energy 15 eV). The dwell times were 150 ms. Argon was used as a collision gas at the optimum collision energy of 15 V. MassLynx 4.1 software was used for data MS control and data gathering. QuanLynx software was used for data processing, peak integration and linear regression.

2.3. Preparation of standard solutions

Stock solutions of both reference standards were prepared by dissolution of entecavir or internal standard entecavir-$C_2{}^{13}$ N^{15} in water at the concentration 1 mg/ml. The stock solutions were further diluted by appropriate dilution media, which corresponded to the composition of mobile phase (75% ACN in water for HILIC, 5% ACN in water for RP separation).

2.4. Preparation of rat urine samples

Rat urine samples were obtained from experiments with the perfused rat kidneys focused on an analysis of quantitative parameters and mechanisms of entecavir renal excretion. Wistar rats (BioTest, Konarovice, Czech Republic) were used as the organ donors. The experiments with animals were approved by the Ethical Committee of the Pharmaceutical Faculty, Charles University in Prague, and were carried out in compliance with the respective Czech laws concerning animal protection.

Blank rat urine samples were always spiked by minimum amount of standard solutions (<5% of the whole prepared volume) to prevent excessive dilution of biological material and misinterpretation of results.

The extraction of entecavir from rat urine was performed by means of solid phase extraction (SPE) on Waters Oasis HLB cartridge (1 ml, 30 mg). The SPE protocol was as follows: (1) the cartridge was activated by 1 ml of acetonitrile followed by conditioning by 1 ml of water. (2) 500 μl of rat urine sample containing internal standard