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An automated, highly sensitive LC-MS/MS assay for the quantification of the opiate antagonist naltrexone and its major metabolite 6β-naltrexol in dog and human plasma

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ABSTRACT

To support animal studies and clinical pharmacokinetic trials, we developed and validated an automated, specific and highly sensitive LC-MS/MS method for the quantification of naltrexone and 6β-naltrexol in the same run. In human plasma, the assay had a lower limit of quantitation of only 5 pg/mL. This was of critical importance to follow naltrexone pharmacokinetics during its terminal elimination phase. The assay had the following key performance characteristics for naltrexone in human plasma: range of reliable quantification: 0.005–100 ng/mL ($r^2 > 0.99$), inter-day accuracy (0.03 ng/mL): 103.7% and inter-day precision: 10.1%. There were no ion suppression, matrix interferences or carry-over.

1. Introduction

Naltrexone, a pure competitive antagonist at opioid receptor sites [1], has been used in the treatment of addiction, including heroin [2], alcohol [3] and gambling [4]. It has also been used for rapid and ultra-rapid opiate withdrawal [5] with success attributed to its antagonistic potency of 2.5 and 12 times that of naloxone and nalorphine, respectively [6].

6β-Naltrexol is the major metabolite in human plasma (Fig. 1). Although it has weaker opioid antagonistic properties, 6β-naltrexol makes a significant contribution to the overall naltrexone effects after oral administration, potentially due to its 10-fold higher systemic exposure compared to its parent.

Several HPLC assays in combination with UV, electrochemical or mass spectrometry detection have been reported for the quantification of naltrexone and, in some cases, its metabolite 6β-naltrexol [7–12]. All of these assays had either similarly complex multi-step offline extraction procedures and/or relatively high lower limits of quantitation of approximately 0.1 ng/mL (LC-MS) or worse (HPLC-UV). This sensitivity may not be sufficient since naltrexone pharmacokinetics is probably best described by a three-compartment model [13] and naltrexone concentrations during the terminal elimination phase are significantly lower than 0.1 ng/mL. In addition, it has been described that naltrexone plasma concentrations below 0.1 ng/mL are still efficacious [14,15].

Recently, an LC-MS/MS assay with a lower limit of quantitation of 10 pg/mL for naltrexone was reported [16]. However, this assay was only developed for dog plasma and did not include the major metabolite 6β-naltrexol that, as mentioned above, may contribute to the clinical activity of naltrexone in humans. This method also used a liquid–liquid extraction in combination with an evaporation step.

To support animal studies and clinical pharmacological trials, we developed and validated an automated LC-MS/MS method for the quantification of naltrexone and its major metabolite 6β-naltrexol within the same run that was sensitive enough to allow for measuring naltrexone plasma concentrations during the terminal elimination phase and after intra-muscular injection of naltrexone sustained release formulations in humans and dogs, the major

Abbreviations: APCI, atmospheric pressure chemical ionization; EDTA, ethylene diamino tetra acetylic acid; LLOQ, lower limit of quantitation; MRM, multiple reaction monitoring; *m/z*, mass/charge; QC, quality control; R.S.D.%, residual standard deviation in %.

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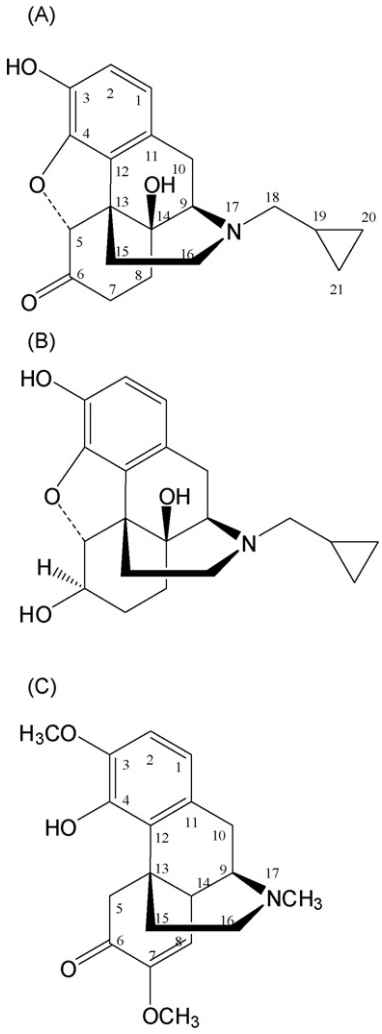

Fig. 1. Structures of (A) naltrexone, (B) 6β-naltrexol and (C) the internal standard sinomenine.

animal model for studying pharmacokinetics after injection of naltrexone sustained release formulations. This assay involved one step-protein precipitation in 96-well plates and a fast online column switching extraction step [17].

2. Materials and methods

2.1. Chemicals and reagents

Solvents and reagents (HPLC-grade methanol and water, formic acid 88%, zinc sulfate) used for sample extraction and as mobile phase were from Fisher Scientific (Fair Lawn, NJ) and used without further purification. Naltrexone and 6β-naltrexol were purchased from Tyco-Mallinckrodt Chemical (St. Louis, MO). The structurally similar opioid sinomenine was used as the internal standard and was purchased from Sigma–Aldrich. The structures of naltrexone, 6β-naltrexol and the internal standard sinomenine are shown in Fig. 1.

2.2. Calibrators and quality control samples

Naltrexone, 6β-naltrexol and the internal standard stock solutions were prepared after three independent weightings. Working solutions for quality control samples, standard curve and the internal standard solution were prepared by dilution of the stock solution in methanol.

Human EDTA plasma samples were obtained from healthy volunteers. Collection of human blood samples from outdated blood bank samples for assay validation and quality control was considered exempt by the Colorado Multi-Institutional Review Board (COMIRB), Denver, Colorado. EDTA dog plasma was obtained from MPI Research (Mattawan, MI) and blank plasma collection was part of an approved animal protocol. The protein precipitation/internal standard solution (methanol/0.2 M $ZnSO_4$, 7:3, v/v, 8 ng/mL sinomenine) was prepared freshly before every extraction. The expiration time for the protein precipitation solution was set to 12 h and the solution was discarded hereafter.

Calibration and quality control samples were prepared by enriching EDTA plasma samples with naltrexone and 6β-naltrexol.

2.3. Sample extraction

The extraction procedure consisted of two steps: a protein precipitation and subsequent online column extraction. The only manual step during the extraction of samples was protein precipitation. Online extraction was based on automated column switching after injection of the supernatant into the HPLC system. Four hundred microliters of protein precipitation solution containing the internal standard (*vide supra*) was added to 100 μL plasma in 96-well plates with 1 mL wells (Agilent Technologies, Santa Clara, CA). Hereafter, the 96-well plates were covered, vortexed (5 min) and centrifuged (4 °C, 13,000 × *g*, 5 min). The 96-well plates were placed into the HPLC autosampler. The online extraction step is described below.

2.4. Equipment

The extracts were analyzed using an LC-MS/MS system in combination with online extraction (LC/LC-MS/MS). The system consisted of the following components: Two G1312A binary pumps, two G1322A vacuum degassers, and a G1316A thermostatted column compartment (all Agilent 1100 series, Agilent Technologies, Santa Clara, CA) in combination with a CTC/PAL thermostatted autosampler (Zwingen, Switzerland) and a 6-port Rheodyne column switching valve (EHMA 055-1431V) mounted on a remote control step motor (Rheodyne, Cotati, CA). The loop volume was 500 μL. The connections of the switching valve are shown in Fig. 2. A Sciex API 5000 triple-stage quadrupole mass spectrometer was used as detector (Applied Biosystems, Foster City, CA). The HPLCs, the switching valve, and the mass spectrometer were controlled by the Analyst software (version 1.4.1).

2.5. LC/LC-MS/MS analysis

One hundred microliters of the sample supernatant was injected onto the online extraction column (12.5 mm × 4.6 mm, 5 μm particle size, C18, Zorbax XDB, Agilent Technologies). The needle of the autosampler was adjusted not to aspire any of the precipitated proteins. Samples were washed with a mobile phase of 20% methanol (containing 0.1% acetic acid) and 80% 0.1% acetic acid in 2 mmol/L ammonium acetate. The flow was 5 mL/min and the temperature for the extraction column was set to 65 °C. After 1 min, the switching valve was activated and the analytes were eluted in the backflush mode from the extraction column onto a 30 mm × 4.6 mm analytical column filled with CN material of 5 μm particle size (Luna, Phenomenex, Torrance, CA). The mobile phase consisted of acetic acid in methanol and 0.1% acetic acid in 2 mmol/L ammonium acetate. The following gradient was run: time 0 min: 40% 0.1% acetic acid + methanol, 4.5 min: 95% 0.1%