Hyphenated LC-MS and NMR Techniques to Identify Metabolites of Bioactive Compounds

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PT-31, 3-(2-chloro-6-fluorobenzyl)-imidazolidine-2,4-dione, is a new heterocyclic pentagonal compound with analgesic and sedative properties, chemically designed to interact with alpha-2 adrenoceptor.¹ During the development of a new drug it is very important to know the biotransformation products formed during the metabolism of the target bioactive compound. Studies of in vitro biotransformation offer many advantages when compared to in vivo studies and it was selected for this study. The aim of this work was to apply LC-MSⁿ and NMR techniques for the identification of in vitro metabolites obtained when liver microsomes from rats were used for the biotransformation of PT-31.

LC separation was performed on a phenyl-hexyl column (150 mm x 2.1 mm; 10 μm) using a mobile phase consisted of methanol/ammonium acetate buffer (5 mmol L⁻¹; pH 5.5) (gradient elution) at a flow rate of 0.4 mL min⁻¹. For the LC-MSⁿ analysis, electrospray ionization (ESI) interface was selected and used in the positive ion mode. The ion trap (IT, Esquire 6000 from Bruker Daltonics) was operated under the following conditions: nebulizer gas 30 psi, dry gas flow 7.0 L.min⁻¹, dry gas temperature 325°C and capillary potential 4500 V. Data acquisition consisted of full MS scan acquired for 100 ms accumulation time, target of 40000 and acquisition range from 100 - 400 m/z in conjunction with data-dependent MS/MS acquisitions on the most intense ions selected from MS-scan spectrum. Post-acquisition of the full-scan MS data was based on the generation of extracted ions chromatograms for expected metabolites. Using the optimized chromatographic conditions, PT-31 eluted at 10 min and, after microsomal incubation using rat liver microsomes, its profile was also monitored by extracted ion chromatogram (m/z 243). Identification of an unknown metabolite in rat liver microsome was achieved successfully by combination of LC-MSⁿ and NMR. It was shown that PT-31 was metabolized to yield 3-(2-chloro-6-fluorobenzyl)-imidazolidine-4-hydroxi-2,4-dione. The identity of this metabolite was established by comparing the LC-MS fragmentation profile and NMR data (¹H and ¹H-¹³C-gHSQC). The NMR experiments were conducted in a Bruker Avance III-600, equipped with a ¹H {¹³C, ¹⁵N} TCI triple resonance cryogenically-cooled probehead.
The $^1$H NMR experiment was based on the 1D version of the NOESY sequence, using water and methanol suppression during the relaxation delay (2.40 s). The spectrum was acquired using 64k data points in a 12,019.23 Hz spectral width, giving an acquisition time of 2.73 s. 256 scans were run to acquire a spectrum with satisfactory signal to noise ratio. Processing was performed using exponential multiplication, applying a line broadening factor (lb) of 0.3 Hz.

LC-MS fragmentation profile revealed the presence of an ion peak at m/z 259, which is consistent with the introduction of a hydroxyl group into the substrate (PT-31). The literature describes several possible pathways for hydroxylation, including aromatic rings and pro-chiral carbons. The MS fragmentation was proposed; however it was not enough to identify the precise position of the hydroxyl group. For this purpose, NMR was the best technique to be applied. A $^1$H-NMR spectrum of the hydroxylated metabolite generated from PT-31 was obtained and compared to the $^1$H-NMR spectrum of PT-31. In comparison to the substrate no significant differences in the chemical shifts of the benzyl group were observed. However, three main changes were observed in the imidazolidine ring, including deshielding of $^1$H from N-H ($1^{st}$position) from 6.09 to 6.71 ppm and the disappearance of CH$_2$ signal ($5^{th}$ position). It was also observed a new singlet signal in 4.64 ppm, which did not exist before the biotransformation process. This signal integrates for 1 H and exhibits a chemical shift consistent with a proton attached to a C-OH group. Due to very low concentration of the obtained metabolite, 2D heteronuclear correlation experiments were not properly acquired. However, only 1D-$^1$H spectra provided data to conclude that the hydroxylation occurred in the $5^{th}$ position of the imidazolidine ring.

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