*Supplementary Material of:*

Brimonidine eye drops at the children reach: a possible foe

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Brimonidine quantification in urine and plasma sample by UPLC-MS/MS analysis

Materials and sample preparation

Brimonidine and internal standard Brimonidine-d4 L-Tartrate (Toronto Research Chemicals) were purchased from Spectra2000 Srl. Brimonidine powder was dissolved in methanol and internal standard Brimonidine-d4 L-Tartrate in water. Internal standard working solutions (100 ng/mL and 2 ng/mL for urine and plasma analysis, respectively) were freshly prepared by diluting the IS stock solution with acetonitrile. Calibrators and Quality Controls (QCs) were prepared in pooled human plasma (anticoagulant K3EDTA) and pooled human urine by standard addition of Brimonidine.

Plasma calibrators were prepared over the range 0-10 ng/mL, with QCs at 0.75, 3 and 7.5 ng/mL for low, mid, and high levels, respectively.

Urine calibrators were prepared over the range 0-500 ng/mL, with QCs at 3, 75 and 375 ng/mL for low, mid, and high levels, respectively.

100 μL of plasma/urine study samples, calibrators, and QCs were added of 300 μL of IS working solution (acetonitrile containing IS 2 ng/mL for plasma samples processing and 100 ng/mL for urine samples processing). After centrifugation, supernatants were transferred in other tubes and then evaporated till dryness. The residues were reconstituted with 100 μL of mobile phase and finally transferred into vials for LC‐MS/MS analysis. 10 μL of sample were injected into the ion source.

UPLC‐ MS/MS analysis for brimonidine quantification in urine and plasma

The LC‐MS/MS system consisted of an ACQUITY™ UPLC™ I‐Class system (comprised of a Binary Solvent Manager (BSM) and a Sample Manager with Flow‐Through Needle (SM‐FTN)) coupled to a Xevo® TQ‐S micro mass spectrometer (Waters Corporation, Milford, MA, USA). The system operated in positive electrospray ionization (ESI+). The run time was 6 min, injection‐to‐injection, using an ACQUITY UPLC® BEH C18 Vanguard pre‐column and an ACQUITY UPLC® BEH C18 2.1 mm x 150 mm, 1.7 μm column (T = 40 °C). Mobile phase was composed of water (A) with 0.1% formic acid and acetonitrile (B) with 0.1% formic acid (B). Flow rate was 0.5 mL/min. LC gradient is elucidated in Supplementary Table S1 (ST1).

 **Table S1.** LC gradient.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Time (min) | Flow rate (mL/min) | %A | %B | Curve |
| 0 | 0.5 | 95 | 5 | initial |
| 1.00 | 0.5 | 95 | 5 | 6 |
| 2.00 | 0.5 | 5 | 95 | 6 |
| 3.00 | 0.5 | 5 | 95 | 6 |
| 3.01 | 0.5 | 95 | 5 | 6 |
| 6.00 | 0.5 | 95 | 5 | 6 |

Parameters referring to MRM functions for the detection of brimonidine are reported in Supplementary Table S2 (ST2).

 **Table S2.** MRM parameters for Brimonidine and internal standard Brimonidine-d4.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Compounds | Transitions (*m/z*) | Dwell (secs) | Cone (V) | Collision energy (eV) |
| Brimonidine (quantifier) | 292.2>212.2 | 0.100 | 50 | 27 |
| Brimonidine (qualifier) | 292.2>249.2 | 0.100 | 50 | 27 |
| Brimonidine-d4 (IS) | 296.2>216.2 | 0.100 | 50 | 27 |

Data were processed using TargetLynx™ XS software (Waters Corporation, Milford, MA, USA). Supplementary Figures (SF1, SF2, and SF3, SF4) show chromatograms related to blank and spiked samples, respectively for urine and plasma sample types.



Figure S1. Chromatographic separation of brimonidine in a urine sample used as a blank sample type. Chromatographic separation of brimonidine (quantifier), brimodine (qualifier), and internal standard Brimonidine-d4 in a urine sample used as a blank urine in the optimization step.



Figure S2. Chromatographic separation of brimonidine in a blank urine spiked sample. Chromatographic separation of brimonidine (quantifier), brimodine (qualifier), and internal standard Brimonidine-d4 in a urine blank sample spiked with Brimonidine 1 ng/mL.



Figure S3. Chromatographic separation of brimonidine in a plasma sample used as a blank sample type. Chromatographic separation of brimonidine (quantifier), brimodine (qualifier), and internal standard Brimonidine-d4 in a plasma sample used as a blank plasma in the optimization step.



Figure S4. Chromatographic separation of brimonidine in a blank plasma spiked sample. Chromatographic separation of brimonidine (quantifier), brimodine (qualifier), and internal standard Brimonidine-d4 in a plasma blank sample spiked with Brimonidine 0.25 ng/mL.