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Development of an Assay for Atropine in Rabbit Plasma by LC/MS/MS and the Impact of Atropine Esterase

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OVERVIEW

Atropine, a plant alkaloid used as an ophthalmic treatment for several eye conditions, is a racemic mixture of S-hyoscyamine and R-hyoscyamine. A quantitative LC-MS/MS assay was developed and validated over the range of 80 pg/mL to 40 ng/mL in rabbit plasma. The current data highlights the approach used to minimize the impact of the enantiospecific and species-specific atropinesterase and describes the methodology used for the achiral quantitation of atropine.

INTRODUCTION

The objective of the program was to develop and validate an LC-MS/MS bioanalytical method for the quantitation of atropine in rabbit plasma in support of TOX/PK studies.

Previously developed methodologies reported good stability in several species, although low extraction recoveries (ca. 50%) were reported in rabbits. While atropine is found to be stable in most mammal plasma, rabbits produce atropinesterase, an enantiospecific enzyme responsible for the rapid hydrolysis of S-hyoscyamine (**Figure 1**). Initial method evaluations revealed that the reported recovery was in fact the result of spontaneous degradation of S-hyoscyamine, prompting further development efforts to confer stabilization.

2,2-dichlorovinyl dimethyl phosphate (DDVP), an organophosphate acetylcholinesterase inhibitor, was successfully used to arrest atropinesterase activity during blood collection efficiently.

Herein, we present experimental details and selected bioanalytical results pertaining to the method development and validation.

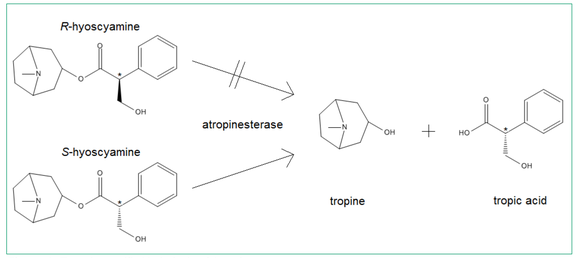

Figure 1. Enantiospecific Hydrolysis of Atropine

METHODS

1. SAMPLE COLLECTION

Blood samples were collected in vacutainer tubes containing 2% dichlorvos pre-fortified in plasma (0.34%) to minimize hemolysis and maintain isotonicity. Samples were kept on ice throughout sample collection and processing with storage at -80°C.

2. EXTRACTION

Plasma samples were pre-chilled on wet ice and extracted by protein precipitation using acetonitrile followed by aqueous dilution of supernatant.

3. CHROMATOGRAPHY

Chromatographic conditions were developed in reversed-phase mode on a Waters™ Acquity HSS T3 column (50 x 2.1 mm; 1.8 µm) with gradient elution, using a 0.1% formic acid (aq) as mobile phase A and acetonitrile as mobile phase B, with a total flow rate of 600 µL/min and column temperature of 40° C. A double gradient strategy was used to mitigate on-column carry-over, as detailed in **Table 1**.

4. MASS SPECTROMETRY

A Shimadzu X2 UPLC system was coupled to an API 5000 (SCIEX) mass spectrometer operated in MRM mode with positive electrospray ionization. Atropine yielded a major fragment at *m/z* 124 that was selected for optimal sensitivity and specificity.

Table 1. Gradient Elution Table

Time (minutes)	Mobile Phase B %
0.00	10
0.30	10
1.30	60
1.31	85
1.80	85
1.81	10
2.20	10
2.60	85
3.00	85
3.01	10

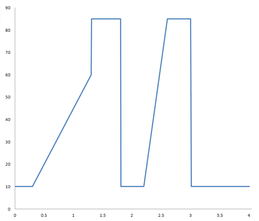

Table 2. MRM Parameters

Compound	Q1 m/z	Q3 m/z	Dwell time (msec)	DP	CE
Atropine	290.2	124.1	150	150	31
Atropine-D5	295.2	124.1	50	150	31

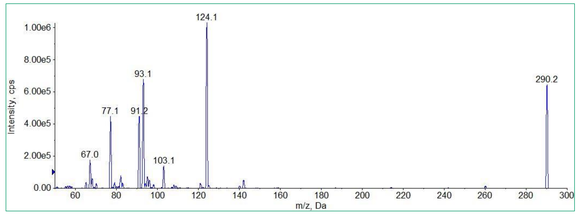

Figure 2. Product Ion Spectrum of Atropine

EXPERIMENTAL DATA

1. RECOVERY FROM PLASMA AND BLOOD

Evaluation of extraction recovery from 12 control rabbit donors sourced commercially revealed variable yields, with most lots >75%, but with three donors at ca. 50% (**Figure 3**). In contrast, extraction recoveries from freshly collected rabbit plasma were ca. 50% from 90% of the donors (**Figure 4**).

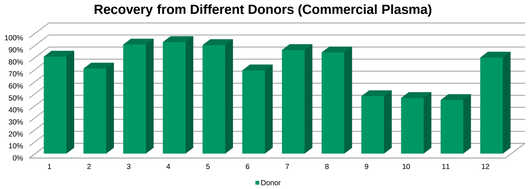

Figure 3. Figure 3. Donor-Dependent Extraction Recovery From Commercial Rabbit Plasma (K2EDTA)

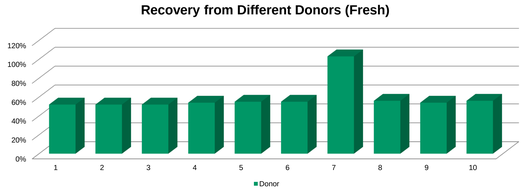

Figure 4. Donor-Dependent Extraction Recovery From Fresh Rabbit Plasma (K2EDTA)

Treating samples with DDVP as described in the sample collection procedure improved the recovery of atropine from those problematic donors. Notably, atropinesterase activity quickly decreased after only a few days of storage, as recovery from untreated blood was quantitative (**Figure 5**).

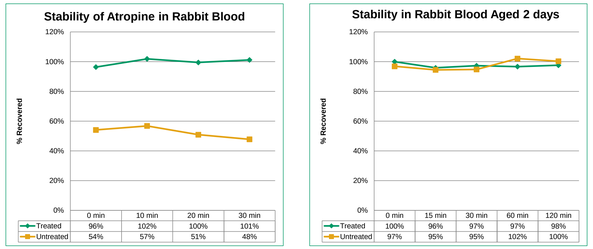

Figure 5. Stability of Atropine in Fresh vs. 2 Days Old Rabbit Plasma (K2EDTA)

2. METHOD EVALUATION

The described methodology yielded satisfactory sensitivity, specificity (Figure 6), and linearity over the targeted range of 80 pg/mL to 40 ng/mL.

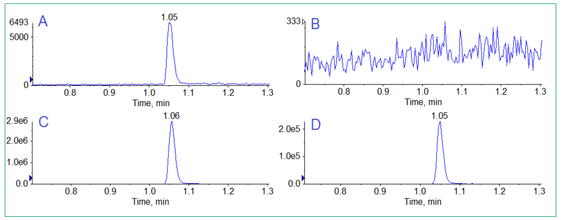

Figure 6. Representative Chromatograms (*A – LLOQ (80 pg/mL) ; B – Blank ; C - ULOQ (40 ng/mL) ; D – Internal Standard*)

Method validation highlights:

- Stability in plasma from treated blood was established for 20 hours at 4°C, with back-calculated concentrations within 5% of nominal.
- Atropine was stable over 4 freeze-thaw cycles and for at least 44 days when stored at -80°C.
- Recovery of both atropine and its internal standard was quantitative.
- Precision and accuracy (P&A) over three analytical batches exhibited % deviations within 8% at LLOQ, low, mid, and high concentration levels, with %CV within 4%.
- No matrix effect was observed from seven individual plasma lots, including 5% hemolyzed.
- Extracted samples were found to be stable for up to 143 hours when stored at 4°C.
- At least one P&A batch represented expected maximal injections (183).

DISCUSSION

The rapid rate of atropine degradation in fresh whole blood required the preservative (0.34% DDVP in rabbit plasma) to be pre-aliquoted in blood collection tubes in order to confer immediate stabilization. Early investigations revealed that atropinesterase activity decreases quickly with blood age and that after only a few days, no degradation was observed. The use of freshly collected rabbit whole blood was, therefore, essential in optimizing the sample collection procedure.

CONCLUSION(S)

A bioanalytical assay was successfully developed and validated according to the ICH M10 guideline for “Bioanalytical Method Validation And Study Sample Analysis.”

The method was used for study sample analysis with an incurred sample reanalysis success rate of 100%, thus confirming the efficacy of the sample stabilization strategy.

CONFLICT OF INTEREST DISCLOSURE
The authors declare no competing financial interest