An Ultra-sensitive, Robust and Fast Assay to Quantify Loteprednol Etabonate and Ketorolac in Human plasma by SLE-UPLC-MS/MS

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INTRODUCTION

Ocular inflammation, occurring in different locations (e.g. ocular adnexa, conjunctiva, et. al.) with different causes (e.g. inflamed pterygium, bacterial infections, et al.), has become a hot topic in ophthalmology. There are two types of drugs for ocular inflammation: steroid drugs and nonsteroidal anti-inflammatory drugs (NSAID). Loteprednol etabonate (LE) is a steroid drug, and Ketorolac is a NSAID, both of which could be used in the treatment of ocular inflammation as a single drug or a combination with other drugs. Therefore, it is critical to have a robust and sensitive method to support the clinical studies. In this paper, we targeted to develop and validate an ultra-sensitive, robust and fast UPLC-MS/MS method to quantify LE and ketorolac in human plasma.

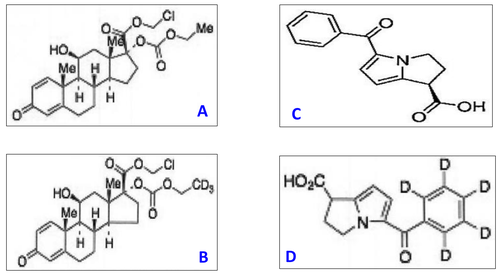

Figure 1: Chemical structure of LE (A), LE-d3 (B), Ketorolac (C) and Ketorolac-d5 (D).

METHOD

Sample Preparation

A 200 µL aliquot of human plasma (K_2 EDTA) containing LE and ketorolac was extracted with internal standards (IS, LE-d3 and ketorolac-d5) by supported-liquid extraction (SLE) using ethyl acetate (EtoAc). The extraction was further dried down under N2 gas and reconstituted with MeOH: H2O (20: 80 v/v) before UPLC-MS/MS analysis.

LC-MS Conditions

HPLC: Shimadzu LC-30AD
Column: Halo 90Å, biphenyl, 2um, 2.1 mm x 75mm, (Part No: 91812-511)
Column temperature: 40ºC
Mobile phase A: 0.1% formic acid in H_2O
Mobile phase B: 0.1% formic acid in acetonitrile (MeCN)

METHOD (cont.)

Needle wash: MeCN/Methanol/IPA/Water (1:1:1:1, *v/v/v/v*)
Flow rate: 0.6 mL/min
Gradient profile: Refer to **Table 1**.

Table 1: HPLC gradient profile

Time (min)	Events	Value
0.50	B conc.	20%
1.00	B conc.	50%
2.00	B conc.	50%
3.50	B conc.	95%
4.50	B conc.	95%
4.51	B conc.	20%
5.00	Stop	

A Sciex API 5500 was used under electrospray ionization mode to monitor LE, ketorolac, and their IS at ion transitions of 467.1→265.3, 256.8→106.1, of 470.3→265.1 and 261.7→111.3, respectively.

RESULTS & DISCUSSION

Sample Preparation Screening (AMEOBA[1])

The level of LE and ketorolac in human plasma was low due to their low dose level and drug administration of eye drop formulation. Thus, the method requires high sensitivity. In order to achieve this, different sample preparation technologies including protein precipitation extraction (PPE), supported-liquid extraction (SLE) and solid-phase extraction (SPE) were compared by following the AMEOBA[1] protocol. The sample preparation results (**Figure 2**) indicated that SLE with EtoAc under acidic conditions provided the highest extraction recovery

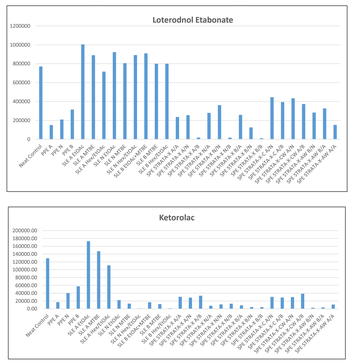

Figure 2: Sample Preparation Screening (AMEOBA) for LE and Ketorolac

RESULTS&DISCUSSION (cont.)

Non-specific Binding (NSB)

For hydrophilic compounds, it is a common practice to evaluate the NSB. Therefore, the NSB was studied in DMSO and dimethyl formamide (DMF) with glass and polypropylene containers. The data showed that LE and KT have severe NSB issues in DMF and glass containers, but no NSB issues in DMSO in polypropylene containers, which were used to prepare stock and working solutions.

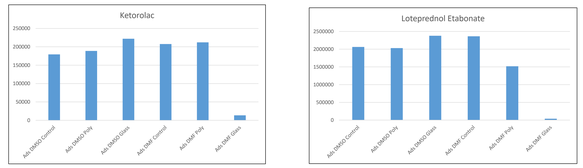

Figure 3: Non-specific Binding for LE and Ketorolac

Linearity and Sensitivity

The quantitative assay was successfully developed and validated within ranges of 0.500/0.100 to 500/100 ng/mL (**Figures 4** and **5**, **Tables 2** and **3**). The inter-assay accuracy (%) for the standard calibrators was 96.1% to 101.5%. The inter-assay precision (%CV) for the standard calibrators was 2.4% to 5.6% over 4 quantitative runs.

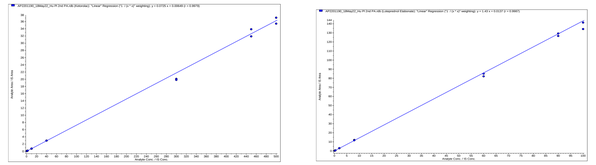

Figure 4: The typical curve for LE and Ketorolac

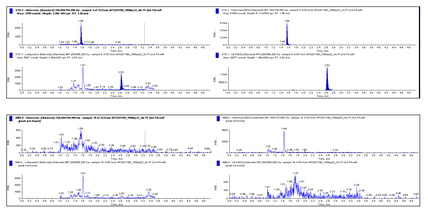

Figure 5: The typical chromatograms

Batch ID	Slope (LE/Ketorolac)	Intercept (LE/Ketorolac)	Correlation Coefficient (LE/Ketorolac)
01	7.25E-02/1.43E0	6.49E-03/1.37E-02	0.9979/0.9987
02	6.98E-02/1.42E0	7.46E-03/1.37E02	0.9979/0.9986
03	7.29E-02/1.35E0	9.44E-03/1.12E-02	0.9994/0.9990
n	3	3	3
Mean	7.17E-02/1.40E-0	7.80E-03/1.29E-02	0.9984/0.9988

Table 2: Curve parameters

RESULTS&DISCUSSION (cont.)

Table 3: Precision and Accuracy for calibration standards

	Concentration (Ketorolac/LE) (ng/mL)							
Batch ID	0.500/0.100	1.00/0.200	2.00/0.400	10.0/2.00	40.0/8.00	300/60.0	450/90.0	500/100
01	0.455/0.0974	0.998/0.216	2.07/0.401	9.64/2.02	40.6/8.40	277/59.3	467/88.3	511/98.7
	0.505/0.0976	1.120/0.209	2.11/0.373	10.3/2.12	40.6/8.14	273/57.3	440/90.3	489/93.7
02	0.519/0.0957	0.911/0.217	2.12/0.418	10.4/2.04	40.9/7.81	289/61.1	461/83.6	492/94.3
	0.513/0.0972	0.918/0.202	2.06/0.411	9.49/2.11	38.0/8.18	293/61.3	505/84.8	496/97.4
03	0.425/0.0986	0.966/0.221	2.06/0.416	9.59/2.03	39.5/7.80	292/60.6	446/89.5	487/99.7
	0.546/0.0949	1.10/0.200	2.16/0.406	9.83/1.99	41.1/8.13	283/55.8	476/90.1	492/97.1
n	6	6	6	6	6	6	6	6
Mean	0.494/0.0969	1.00/0.211	2.10/0.404	9.88/2.05	40.1/8.08	285/59.2	466/87.8	490/100
SD	0.0449/0.00135	0.0896/0.0857	0.0403/0.0165	0.385/0.0519	1.18/0.232	8.2/2.24	23.3/2.87	8.60/2.38
CV(%)	9.1/1.4	9.0/4.1	1.9/4.1	3.9/2.5	2.9/2.9	2.9/3.8	5.0/3.3	1.8/2.4
Accuracy (%)	98.8/96.9	100.0/105.5	105.0/101.0	98.8/102.5	100.3/101.0	95.0/98.7	103.6/97.6	98.0100.0

Precision and Accuracy

Spiked quality control (QC), sample precision, and accuracy were demonstrated at n=18 at the lower limit of quantification (LLOQ-QC, 0.500/0.100 ng/mL), at low QC (LQC, 1.50/0.300 ng/mL), medium QC (MQC, 15.0/3.00 ng/mL), and high QC (HQC, 400/80.0 ng/mL) concentrations over three validation runs. The LLOQ and other QCs were within 20% and within 15%, respectively (**Table 4**).

QC ((Ketorolac/LE) (ng/mL)	n	Mean	S.D.	%CV	Accuracy
0.500/0.100	18	0.481/0.0964	0.0457/0.00572	9.5/5.9	96.2/96.4
1.50/0.300	18	1.37/0.293	0.0743/0.0128	5.4/4.4	91.3/97.7
15.0/3.00	18	13.9/3.02	5.4/0.0853	7.6/2.8	92.7/100.7
400/80.0	18	386/79.8	91.3/4.70	6.9/100.7	96.5/99.8

Table 4: Inter-assay precision and accuracy for QC samples

Internal Standard Response

As a reference for assay performance, the IS responses (peak areas) of the calibration standards and LLOQ-QC, LQC, MQC, and HQC samples from all of the inter-assay precision and accuracy batches were evaluated (**Table 5**).

Internal standard (IS):	IS response variation (%CV):
Ketorolac-d5	4.62
LE-d3	4.56

Table 5: Internal standard response

CONCLUSIONS

- A sensitive and fast LC-MS/MS method for the quantification of LE and Ketorolac was developed.
- The method was validated as linear, accurate, precise and reproducible. It can be used to determine the concentration of LE and ketorolac as low as 0.100 and 0.500 ng/mL using 200 µL of sample.

Reference

1. M. Meng, L. Wang, T. Voelker, S. Reuschel, K.V. Horne, P. Bennett. A systematic approach for developing a robust LC-MS/MS method for bioanalysis. Bioanalysis. 2013 Jan;5(1):91-115. doi: 10.4155/bio.12.295.