A Sensitive, High-Throughput Assay to Quantify Loteprednol Etabonate and Ketorolac Tromethamine Using SLE-UPLC-MS/MS in Human Urine Treated with SDS

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OBJECTIVE

The objective of this study was to evaluate the nonspecific binding (NSB) of loteprednol etabonate (LE) and ketorolac tromethamine (KT) in human urine, and then develop and qualify a sensitive, high-throughput method using ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) to measure their concentrations.

INTRODUCTION

NSB is one of the most common issues for hydrophilic compounds in a urine sample since the matrix is lacking of proteins and lipids that can bind to the analytes or solubilize lipophilic analytes. LE and KT are hydrophilic compounds that are used to treat ocular inflammation. When a rapid and robust assay is being developed to quantify analytes in a urine sample, NSB needs to be taken into consideration at the beginning of method development. In this poster, we discussed a general strategy for handling NSB issues. With a good understanding of the chemical nature of the analytes, we have successfully developed and qualified a sensitive, high-throughput, quantitative UPLC-MS/MS assay to measure the concentrations of LE and KT in human urine.

METHOD

Sample Preparation

To prevent NSB and remove interference from the matrix, a 200-µL human urine sample, which was treated with 1% sodium dodecyl sulfate (SDS), was extracted with internal standard (IS) using ethyl acetate (EtOAc) through supported-liquid extraction ([SLE], Strata® DE SLE 400-µL, 96-well plate), and then dried down with nitrogen gas using methanol/water (MeOH/H_2O, 20:80, *v/v*) as the reconstitution solvent before UPLC-MS/MS analysis.

LC-MS Conditions

HPLC: Shimadzu LC-30AD
Column: Waters ACQUITY UPLC™ BEH C8 column (130Å, 1.7 µm, 2.1 × 50 mm)
Column temperature: 50°C
Mobile phase A: 0.1% formic acid in H_2O
Mobile phase B: 0.1% formic acid in acetonitrile (ACN)
Needle wash: ACN/MeOH/IPA/acetone (1:1:1:1, *v/v/v/v*)

METHOD (cont.)

Flow rate: 0.8 mL/min
Gradient profile: Refer to **Table 1**.

Table 1. HPLC gradient profile

Time (min)	Mobile Phase A Percentage	Mobile Phase B Percentage
0.00	80%	20%
0.50	30%	70%
1.00	5%	95%
2.50	5%	95%
2.55	80%	20%
3.00	80%	20%

A SCIEX API 5500 triple quadruple mass spectrometer was used in electrospray ionization (ESI) mode with positive ions to monitor analytes LE and KT, and their internal standards (IS), at ion transitions of 467.1→265.3 and 256.8→106.1, and of 470.3→265.1 and 261.7→111.3, respectively.

RESULTS

Nonspecific Binding (NSB)

A comprehensive experiment was designed to assess the NSB of LE and KT in human urine, and the result showed ~50% of LE lost after five transfers in polypropylene container. Pretreatment like adding anti-adsorptive additives to urine samples in clinical study is the typical way to overcome the NSB issue. In this study, various additives, including BSA (bovine serum albumin), CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), Triton™ X100, and SDS, were evaluated. (**Table 2**)

Table 2. Various additives screening for preventing NSB

Difference in analyte counts after 5 transfers		
Additive	**LE**	**KT**
BSA 1%	-23%	-3%
BSA 0.1%	-15%	-14%
CHAPS 0.1%	13%	-3%
CHAPS 0.01%	-82%	-70%
Triton x100 0.5%	-3%	-12%
Triton x100 0.05%	6%	-8%
SDS 0.5%	-6%	-8%
SDS 0.05%	-15%	53%

RESULTS (cont.)

Additives BSA, CHAPS, Triton X100, and SDS were analyzed by MS and then separated and diverted to waste, based on their retention times. Among these additives, SDS demonstrated to be the most efficient one to prevent sample loss. The SDS concentrations were further optimized, and the result revealed that 0.2–2% SDS effectively prevented NSB. (**Table 3**).

Table 3. SDS concentration optimization

Difference in analyte counts after 5 transfers		
SDS Concentration	**LE**	**KT**
5%	-63%	-23%
2%	-18%	-8%
1%	8%	-4%
0.5%	3%	10%
0.2%	-25%	10%
0.1%	-14%	-37%

Besides the NSB issue, MS challenges like low sensitivity, high background noise, and interference peaks were also encountered. In order to increase sensitivity, different sample preparation technologies were compared, and supported liquid extraction (SLE) provided the highest extraction recovery. Different chromatographic parameters (e.g., stationary phase, mobile phase, and LC gradient) and mass spectrometer parameters (e.g., Q1/Q3 transition) were optimized to eliminate the issues of high MS background noise and interference peaks (**Figure 2**).

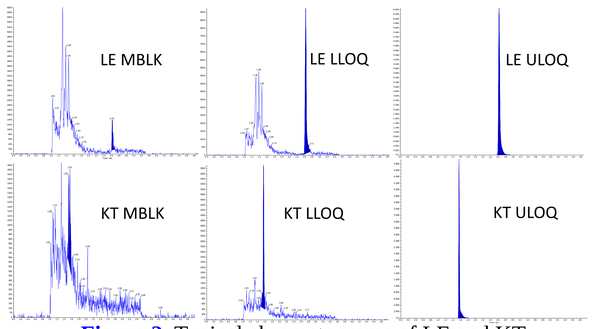

Figure 2. Typical chromatograms of LE and KT

Linearity and Sensitivity

The quantitative assay was developed and qualified within a range of 0.100 to 100 ng/mL for LE and 0.500 to 500 ng/mL for KT (**Figure 3** and **Table 4**). The inter-assay accuracy (%) for the standard calibrators was 90.0% to 105.8% for LE and 98.0% to 102.5% for KT. The inter-assay precision (%CV) for the standard calibrators was 1.9% to 5.2% for LE and 2.2% to 6.3% for KT over 3 quantification runs.

RESULTS (cont.)

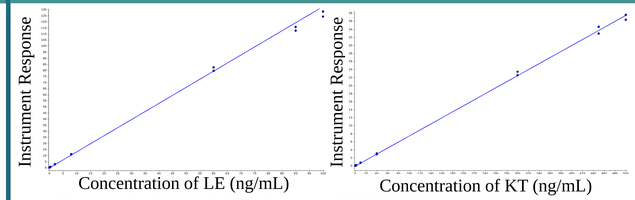

Figure 2: Typical LE (left) and KT(right) calibration curves

Table 4. Calibration curve parameters for LE and KT

	Calibration Curve Parameters for LE			Calibration Curve Parameters for KT		
Batch ID	**Slope**	**Intercept**	**Correlation Coefficient (*R*)**	**Slope**	**Intercept**	**Correlation Coefficient (*R*)**
01	1.33E+00	2.17E-02	0.999	7.51E-02	2.97E-03	0.9991
02	1.34E+00	6.20E-03	0.9972	7.61E-02	7.98E-04	0.998
03	1.33E+00	1.81E-02	0.9984	7.33E-02	2.21E-03	0.9996
n	3	3	3	3	3	3
Mean	1.33E+00	1.53E-02	0.9982	7.48E-02	1.99E-03	0.9989

Precision and Accuracy

Precision and accuracy were evaluated using spiked quality control (QC) samples (n=18) at the lower limit of quantification QC (LLOQ-QC, 0.100 ng/mL for LE and 0.500 ng/mL for KT), low QC (LQC, 0.300 ng/mL for LE and 1.50 ng/mL for KT), medium QC (MQC, 3.00 ng/mL for LE and 15.0 ng/mL for KT), and high QC (HQC, 80.0 ng/mL for LE and 400 ng/mL for KT) concentrations over 3 quantification runs. The precision and accuracy of LLOQ and other QCs were within 20% and within 15%, respectively (**Table 5** and **Table 6**).

Table 5: Inter-assay precision and accuracy for LE QC samples

QC (ng/mL)	*n*	Mean	SD	%CV	Accuracy
0.100	18	0.101	0.00490	4.9	100.7
0.300	18	0.319	0.00948	3.0	106.3
3.00	18	3.25	0.0599	1.8	108.3
80.0	18	81.6	2.10	2.6	102.0

Table 6: Inter-assay precision and accuracy for KT QC samples

QC (ng/mL)	*n*	Mean	SD	%CV	%Accuracy
0.500	18	0.459	0.0334	7.3	91.8
1.50	18	1.39	0.0877	6.3	92.7
15.0	18	14.5	0.660	4.6	96.7
400	18	392	13.4	3.4	98.0

CONCLUSIONS

- The nonspecific binding issue of LE and KT in human urine was evaluated and solved.
- A sensitive and fast SLE-UPLC-MS/MS method for the quantification of LE and KT in human urine was successfully developed and qualified.