The Determination of Citric Acid in Human Urine by LC/MS/MS

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Introduction

Citrate is indicated for the management of renal tubular acidosis with calcium stones. The quantitation of citric acid is challenging due to the high endogenous level of citric acid in human urine. Here we describe a very simple, sensitive and cost effective LC/MS/MS method for the determination of citric acid in human urine. The urine used to make calibration standards and quality control samples was citric acid depleted using the enzymatic conversion in dialysis bags. Citric acid and iso-citric acid was chromatographically separated. This method offered good precision and accuracy and was successfully applied for the pharmacokinetic and bioequivalency studies of 10 mEq Extended-Release Tablets.

Calibration standards and quality control samples were prepared by spiking known amount of citric acid to citric acid depleted human urine. Citric acid and internal standard, citric acid-d_4, were diluted and then chromatographed using reversed phase HPLC. The analytical column was a Prevail Organic Acid 3µ (150 x 4.6 mm). An API3000 or API4000 with ESI source was operated in negative ion-mode using transition m/z 191.0→111.0 for citric acid and m/z 195.0→113.0 for citric acid-d_4.

No matrix interference and matrix effects were observed. Acceptable intra-day and inter-day assay precision and accuracy were observed over a linear range of 5.00 to 1000 mcg/mL (low curve) and 50.0 to 2000 mcg/mL (high curve).

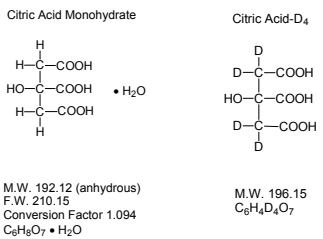

Figure 1. Structure of Citric Acid and Citric Acid-D_4

Method

Since citric acid is an endogenous compound in human urine, the urine used to make standards and quality control samples was citric acid depleted by means of dialysis. Dialysis is a method for removing small molecules from a solution by diffusion through a semi-permeable membrane. Large molecules are retained within the dialysis bag while small molecules and ions pass through. The enzymatic conversion of citrate to oxaloacetate and acetate was accomplished by citrate lyase, a bacterial enzyme from *Aerobacter aerogenes*. The enzyme used for conversion also contains oxaloacetate decarboxylase. This decarboxylates part of the oxaloacetate formed from citrate with citrate lyase to pyruvate. The reaction is shown as follows:

$$Citrate \xrightarrow{Citrate\ \ Lyase} Oxaloacetate + Acetate$$

$$Oxyloacetate \xrightarrow{Oxyloacetate\ \ Decarboxylase} Pyruvate + CO_2$$

The enzymes were loaded in the dialysis bag. Citric acid is a small molecule and easily diffuses into the dialysis bag. Then the enzymes digested the citric acid molecules inside the bag. The citric acid molecules in the urine are gradually diffused into the dialysis bag due to concentration gradient and continuous mixing resulting in a urine matrix depleted of citrate.

The citric acid depleted urine was screened by LC/MS/MS to confirm the absence of interference at the retention time of the citric acid. This urine was then spiked with known amount of citrate acid to prepare calibration standards and quality control samples.

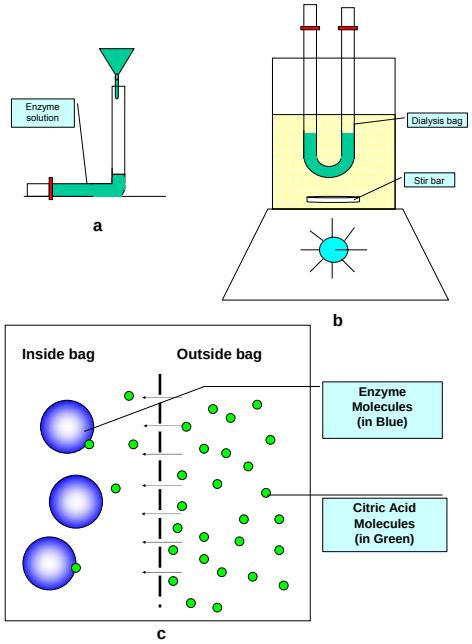

Figure 2. Citric Acid Depletion Process: a) Fill the dialysis bag with enzymes; b) Seal the dialysis bag and put it in the urine; c) Citric acid depletion in the dialysis bag.

Liquid Chromatography

Shimadzu 10ADvp HPLC system
Column : Prevail Organic Acid, 150 x 4.6 mm, 3 µ, Part No. 88655
Mobile Phase A : 0.5% Formic acid /MeOH (90/10)
Mobile Phase B : methanol
Gradient Program : Flow rate (0.50 mL/min)

Time (min)	0.01	5.00	5.01	7.00
% of B	10.0	50.0	10.0	stop

Mass Spectrometry

Applied Biosystem/MDS-Sciex API 3000 or API 4000
Polarity : negative
Scan type : Multiple Reaction Monitoring
Citric acid : 191.0→111.0 m/z
Citric acid-d_4(I.S.) : 195.0→113.0 m/z

Sample Preparation Process:

Transfer 100 mcL urine sample into glass tubes, add 200 mcL working internal standard (WIS, 10 mcg/mL Citric Acid-d_4), add 5 mL 40 mM ammonium formate, *pH 3.0* solution. Mix well. Transfer a portion into injection vials.

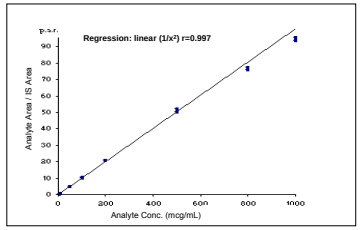

Figure 3. Calibration curve 5 to 1000 mcg/mL for Citric Acid in Human Urine

Results

The method was fully validated over a range of 5.00 to 1000 mcg/mL and 50.0 to 2000 mcg/mL with weighted linear regression (1/x^2). The correlation coefficients for first three validation batches were 0.997 or better (Figure 3).

Figures 4 through 7 show typical chromatograms of extracted lower and upper limits of quantitation (LLOQ 5.00 mcg/mL, ULOQ 1000 mcg/mL), an extracted blank matrix, and an extracted patient sample. No carryover or interference was observed in the chromatogram of the blank matrix.

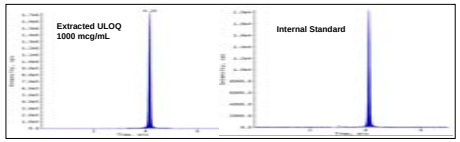

Figure 4. Chromatogram of an extracted ULOQ sample (1000 mcg/mL).

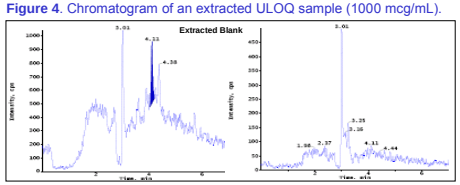

Figure 5. Chromatogram of an extracted blank sample

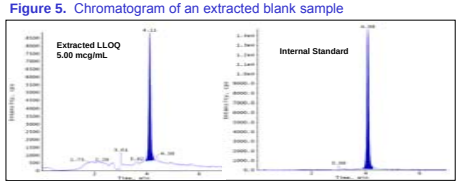

Figure 6. Chromatogram of an extracted LLOQ sample (5.00 mcg/mL).

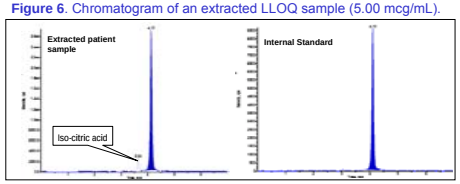

Figure 7. Chromatogram of an extracted patient sample.

Table 1 Summary results of inter-batch, intra-batch precision and accuracy.

Batch		5.00 mcg/mL LLOQ	15.0 mcg/mL Low QC	400 mcg/mL Mid QC	750 mcg/mL High QC
1	mean	5.47	16.3	399	742
	sd	0.545	1.1	8.89	14.2
	cv [%]	10	6.8	2.2	1.9
	bias [%]	9.5	8.4	-0.3	-1.1
2	mean	4.46	14.2	389	724
	sd	0.449	0.734	12.2	26.5
	cv [%]	10.1	5.2	3.1	3.7
	bias [%]	-10.8	-5.6	-2.7	-3.4
total	n	12	12	12	12
	mean	4.97	15.2	394	733
	sd	0.712	1.41	11.3	22.2
	cv [%]	14.3	9.3	2.9	3
	bias [%]	-0.7	1.4	-1.5	-2.3

Table 2. Summary of Stability Results for Citric Acid.

Matrix stability	
Freeze-thaw stability	4 cycles
Bench-top stability	24 hours
Refrigerator stability	27 hours
Long-term stability	47 days
Extracted Matrix stability	
Autosampler stability at room temperature	97 hours
Refrigerated extract stability	97 hours
Individual sample reinjection at room temp.	Reproducible
Whole batch reinjection Integrity	5 days
Solution stability	
Primary solution stability at 4 °C	34 days
Primary solution stability at RT	24 hours

Stability results for citric acid are listed in **Table 2**. Selectivity was tested by adding 100 mcL citric acid spiking solution to 900 mcL of 8 individual lots of human urine to prepare the matrix effect QCs, which are 4 times of LLOQ level over the endogenous level. The precision of response factor in eight different lots of urine matrix was determined. (**Table 3**). No interference or matrix effects were observed.

Table 3. Matrix Effect for Citric Acid in Human Urine.

Sample ID	Spiked Conc mg/mL	Urine Conc mcg/mL	Total Conc mcg/mL	Analyte Area	Response Factor (Rf)	Avg IS Area	Avg Rf/IS Ratio
ME1	2.00	0.00	200.0	70758	354	39733	0.00890
ME2	2.00	17.6	215.8	76312	354	38359	0.00922
ME3	2.00	0.00	200.0	72996	365	36509	0.01000
ME4	2.00	8.37	207.5	76689	370	36702	0.01007
ME5	2.00	0.00	200.0	76981	385	38308	0.01005
ME6	2.00	0.00	200.0	84065	420	38013	0.01106
ME7	2.00	0.00	200.0	78221	391	38406	0.01018
ME8	2.00	654	788.6	350246	444	42258	0.01051
	Mean				387	38536	0.01000
	SD				31.29	1756	0.00066
	%CV				8.1	4.6	6.6

Response Factor (Rf) = Analyte Area/Total Conc. (mcg/mL).

Conclusion

Validation results obtained in this study indicate that a robust and reliable method has been established for the measurement of citric acid in human urine by LC/MS/MS. This validated method is suitable for determination of citric acid in the human urine samples.