

HPLC ANALYSIS OF HUMAN URINE FOR OXALATE CONTENT

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ABSTRACT

Objective: In the present communication, development and validation of reverse phase-high performance liquid chromatography method have been carried out for estimation of oxalate content in the urine of human volunteers with recurrent kidney stone disease and healthy status.

Methods: The analysis of oxalic acid has been carried out on KYA TECH HiQ Sil C₁₈HS column using a mobile phase of methanol: 0.001 N acetic acid in water (50:50, v/v) with a flow rate of 1 ml/min and detection wavelength, 237 nm.

Results: Analysis of oxalate content was carried out using single point calibration method with retention at 2.705 min with good resolution parameters. Urine sample collected from kidney stone patients and healthy volunteers over the period of 24 h were analyzed and it has been found that concentration of oxalate in healthy volunteers is less than 12 µg/ml whereas that in case of kidney stone patients is in the range of 39-151 µg/ml and this data can be utilized for further interpretations about oxalate content in healthy and kidney stone diseased volunteers. This method was validated as per united states food and drug administration (USFDA) guidelines by the study of accuracy, precision, linearity, range, selectivity, the lower limit of quantitation, extraction recovery studies and stability studies for determining oxalate content in the urine of human volunteers. As relative standard deviations of oxalate content estimated are less than 5 percent, the method can be claimed accurate, precise, sensitive and selective for determining oxalate content in the urine of human volunteers.

Conclusion: The results are satisfactory, proving the effectiveness of the method for analysis of oxalate content from other biological fluids with few optimizations.

Keywords: HPLC, Kidney stone, Oxalate, Urine, USFDA

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INTRODUCTION

Calcium oxalate is a chemical compound that forms envelope-shaped crystals, known in plants as raphides. It is a major constituent of human kidney stones, and also found in beer stone, a scale that forms on containers used in breweries. Its chemical formula is CaC₂O₄ or Ca(COO)₂. Calcium oxalate crystals in the urine are the most common constituent of human kidney stones, and calcium oxalate crystal formation is also one of the toxic effects of ethylene glycol poisoning [1]. But kidney stone will occur in hypercalcemia or hyperoxalemia. It will be a big question as various factors like age, gender, diet will be responsible for hyperoxaluria in the form of kidney stones [2-4]. In hypercalcemia or hypoxaemia, excess calcium or oxalate will be present in urine, but the formation of calcium oxalate crystals also depends upon various types of factors like the saturation of urine with both components [5]. About 10% of people will experience nephrolithiasis in their lifetime, and about 70% of those will have recurrences. About 80% of stones are calcium based, and out of these, 80% of those are calcium oxalate stones [6].

Various types of factors like pH, sample collection process parameters, the concentration of ascorbic acid are found to affect the estimation of urinary oxalate [7]. Hence gas chromatographic [8-10], high performance liquid chromatographic [11-13], ion pair chromatographic [14-15] methods have been developed for analysis of oxalate from biological fluids. The various other enzymatic [16-21], colorimetric [22, 23] and isotachopheresis [24, 25] methods have been developed for analysis of oxalate from biological fluids and in the presence of oxalate concentration altering bio-molecules like ascorbic acid. Among all these methods, oxalate oxidase (OO) method was most widely used as it was developed in the form of a colorimetric kit which will give accurate, robust and uniform result when you analyze the same at any centre over the entire globe.

Various laboratories have developed their high-performance liquid chromatography-based using a different setup (column, eluant, detector, retention time, etc) for estimation of oxalate content from native urine and urine spiked with oxalate and comparative profile of these methods has been evaluated by Zerwekh [26] Hesse [27], suggesting need of universal method for bio-analysis of oxalate. Most of the reported methods involve pre-analysis sample processing to achieve maximum oxalate content for analysis.

Hence there is need of simple method which will be universal for estimation of oxalate content from biological matrices otherwise such method needs relative standardization with previously developed method [28]. In this communication, we have proposed new high-performance liquid chromatography (HPLC) method for estimation of oxalate from biological matrices without pre-analysis sample preparation which would be simple, accurate, precise, sensitive and selective for the same and useful over the entire globe.

MATERIALS AND METHODS

Materials

Chemicals

Acetonitrile, oxalic acid, methanol, acetic acid and distilled water (Loba Chemie Pvt. Ltd. Mumbai, India) of HPLC grade were used. The urine samples of healthy and kidney stone diseased human volunteers were collected in department of urology, Government Medical College, and Endo-Urology Centre, Gune's Nirmal Nursing Hospital, Kolhapur, Maharashtra, India with all ethical procedure and secured permission for this research work from Institutional Ethical Committee (IEC), National Centre for Cell Science (NCCS), Pune (Approved in thirteen meeting of IEC of NCCS, Pune).

Instruments

HPLC system

The HPLC system used was a PC based Jasco series comprising of a pump PU-2080 (Dual piston with gear is driven pump) and a UV-2070 detector [29]. The UV detector used in this HPLC system was a Czerny turner mount monochromator with deuterium lamp as the light source. Manual injections were carried out using a rheodyne injector with a fixed 20 µl external loop. The chromatographic and the integrated data were recorded using a Hercule 2000 (interface) computer system. Data processing was carried out using Borwin software of 1.5 version.

UV-visible double beam spectrophotometer

Spectrophotometric analysis was carried out on Jasco model V-530 UV-Visible double beam high-speed scanning spectrophotometer with a single monochromator with a 1200 grooves/mm concave grating. The detector was silicon photodiode (S1337). Light sources used were deuterium lamp (190 to 350 nm) and a halogen lamp (330 to 1100 nm). The instrument had wavelength accuracy of ± 0.3 nm and baseline stability of ± 0.001 Abs/h.

Methods

Preparation of standard solutions of oxalic acid

Fifty mg of HPLC grade pure oxalic acid was weighed accurately. It was then transferred to 50 ml volumetric flask and volume was made up to the mark with pure methanol having concentration 1000 µg/ml. It was then sonicated and used for method development. After method optimization, a solution of oxalic acid was prepared in mobile phase (methanol: 0.001N acetic acid in water (50:50, v/v)) of same strength (1000 µg/ml).

Selection of wavelength of analysis

The standard stock solution was diluted to 10 µg/ml. The wavelength of analysis of oxalic acid was selected by analyzing 10 µg/ml solution of same in pure methanol on UV-Visible double beam spectrophotometer over 200 to 400 nm wavelength range. The wavelength of 237 nm was selected having maximum absorbance.

Collection of urine samples

All study population inclusion of only male candidates, a total of 24 recurrent stone patients and 15 healthy subjects were included in the study population. The exclusion criteria were an active urinary

infection, previous intestinal surgery, and intestinal disease. Patients who have already confirmed the nephrolithiasis by ultra-imagines; and none of the patients had received antibiotics or probiotic preparations in the 3 mo before enrollment. Subjects voluntarily accepted the study with consent. Institutional ethics committee approval had been obtained before the commencement of the study (other data). The urine samples of human volunteers were collected for method development and analysis over a period of 24 h. as claimed by Naim [30] and Curhan [31]. These urine samples were refrigerated until its analysis. Neither preservative nor any processing was carried out on collected urine sample. It was then processed for analysis to determine oxalate content by HPLC.

Chromatographic method development for oxalate analysis in urine

The sample solution of oxalic acid in urine for method development was prepared by mixing 0.1 ml of stock solution of oxalic acid in the mobile phase and 2 ml of urine of healthy human volunteer and diluting it to 10 ml with mobile phase. This solution was then injected to a chromatographic system with KYATECH HiQ Sil C₁₈HS column using rheodyne injector at a flow rate of 1 ml/min and wavelength of analysis, 237 nm. The oxalic acid was resolved at 2.7 min with good retention parameters under above chromatographic conditions. As the concentration of oxalic acid was calculated by single point calibration method, the same sample solution was then injected under similar conditions for 5 more times, and area of the oxalic acid peak was recorded using software. The mean of area of six readings of oxalic acid in urine was calculated, and concentration of oxalic acid in urine patient was calculated using following formula,

$$\text{Oxalic acid content } (\mu\text{g/ml}) = \frac{\text{area of oxalate peak}}{\text{mean of area of 6 readings}} \times 10$$

The concentration of oxalic acid in mg/x ml of urine was calculated by multiplying above reading by dilution factor and x.

Analysis of oxalate in quality control samples and in urine of volunteers

The quality control samples were prepared from standard stock solution and urine of healthy volunteers and analyzed by the same procedure is given above. The results are reported in table 1.

Table 1: Results of analysis of quality control samples, linearity and extraction recovery studies of oxalate

Analysis of quality control samples and extraction recovery studies						
Concentration (ng/ml)	% Concentration estimated mean ^a ±SD ^b		% Recovery estimated mean ^c ±SD ^d			
50	85.65±1.4592		90.70±2.4174			
150	87.76±1.4208		89.16±1.8503			
300	87.84±1.0597		92.21±2.1824			
Linearity and range study						
Range in ng/ml	Slope	Intercept	SE ^d of slope	SE ^d of intercept	Correlation coefficient	
50-300	3.357×10 ⁻³	5.217×10 ⁻²	3.024×10 ⁻⁵	4.781×10 ⁻⁴	0.9981	

^aAverage of five determinations, ^bStandard deviation, ^cAverage of four determinations, ^dStandard error

Analysis of urine of kidney stone patients and healthy volunteers for oxalate content was carried out by the method explained above without adding 0.1 ml of stock solution of oxalic acid in the mobile phase. The results of analysis are reported in table 2.

RESULTS AND DISCUSSION

The brief study of various types of chromatographic, spectroscopic methods emphasized the need of simple, accurate and universal method used for analysis of oxalate in a biological fluid, especially urine. The method was found to be satisfactory for analysis of oxalate from urine samples of human volunteers as after observing chromatogram; it can be concluded that most of other interfering components in human urine were not retained in optimized mobile phase except few. Even though, colorimetric or enzymatic methods were widely used for analysis, developed HPLC method was found to

be simple as it required a minimum number of steps compared to these methods.

The sample collection of urine from kidney stone diseased patient and healthy volunteers was carried out over a period of 24 h as all of the stones from patients contain calcium and oxalate and urinary saturation with oxalate will occur over this period through metabolism [3]. Since, every metabolic workup includes measurement of calcium and oxalate concentrations in a 24 h urine collection, together with other key analytes [30, 31].

The chromatographic separations were performed on a 5 mm KYATECH HiQ Sil C₁₈HS column (250 mm × 4.6 mm i.d., 5 µm particle size), operating at ambient temperature. The standard stock solution of oxalic acid was prepared in pure methanol having concentration 100 µg/ml. The method development was initiated by analyzing 10

$\mu\text{g/ml}$ solution of oxalic acid in methanol on HPLC system. The spectrum of pure oxalic acid was not resolved with good retention parameters (capacity factor, resolution, tailing factor, the number of theoretical plates, etc) [32]. The oxalic acid was resolved in methanol: water (50:50, v/v) mobile phase with good retention parameters. One ml of oxalic acid solution was then mixed with 1 ml of healthy human urine, and this mixture was diluted to 10 ml with methanol: water (50:50, v/v). This mixture was then injected to HPLC system, and chromatogram was recorded, and it was not resolved with good retention parameters. After several trials, methanol: 0.001 N acetic acid in water (50:50, v/v), the mobile phase has resolved oxalic acid in a urine sample at a retention time of 2.705 ± 0.005 min with good retention parameters (fig. 1). Thus oxalic acid in urine sample was possible to quantify on $5 \mu\text{m}$

KYATECH HiQ Sil C_{18} HS column (250 mm \times 4.6 mm i.d.) at a flow rate of 1 ml/min, at a wavelength of 237 nm using methanol: water (50:50, v/v) 0.001 N acetic acid mobile phase. The results of analysis obtained after analysis of urine sample from normal and healthy volunteers were found to be satisfactory and reliable, but this method needs to be validated. Hence validation of method has been carried out by respective guidelines.

In most of the methods, sample preparation for oxalate content required pre-acidification by acetic acid [26, 27] but in the present communication, we have used acetic acid as part of mobile which makes the method as there is no need of sample pre-processing step. This acetic acid has increased the concentration of oxalate which has been retained over the stationary phase.

Table 2: Result of determination of oxalate content in urine of healthy (HLT) and kidney stone diseased (KSD) volunteers

Volunteer code	Number of stones	Concentration in $\mu\text{g/ml}$	Volunteer code	Number of stones	Concentration in $\mu\text{g/ml}$
KSD_1	5	140.6024	KSD_30	4	50.4328
KSD_2	6	70.9542	KSD_31	6	120.2586
KSD_3	5	40.0254	KSD_32	5	60.9981
KSD_4	4	60.1502	KSD_33	4	50.6925
KSD_7	7	80.8047	HLT_1	0	2.0875
KSD_9	5	100.6679	HLT_2	0	3.8035
KSD_10	5	70.4897	HLT_3	0	0.5784
KSD_12	8	50.9875	HLT_4	0	2.7721
KSD_13	4	130.8206	HLT_5	0	2.1589
KSD_14	4	90.5782	HLT_6	0	1.9029
KSD_20	5	80.2496	HLT_7	0	0.8565
KSD_21	6	150.2171	HLT_8	0	11.4752
KSD_22	4	50.6745	HLT_9	0	0.8812
KSD_23	5	50.8762	HLT_10	0	0.4785
KSD_24	4	70.9864	HLT_11	0	10.1217
KSD_25	3	120.9987	HLT_12	0	6.7485
KSD_26	5	60.3574	HLT_13	0	0.8979
KSD_27	6	70.6348	HLT_14	0	3.2486
KSD_28	5	60.0269	HLT_15	0	0.0573
KSD_29	4	50.3985			

The sample chromatograms of analysis of healthy volunteer and kidney stone patient urine are shown in fig. 1.

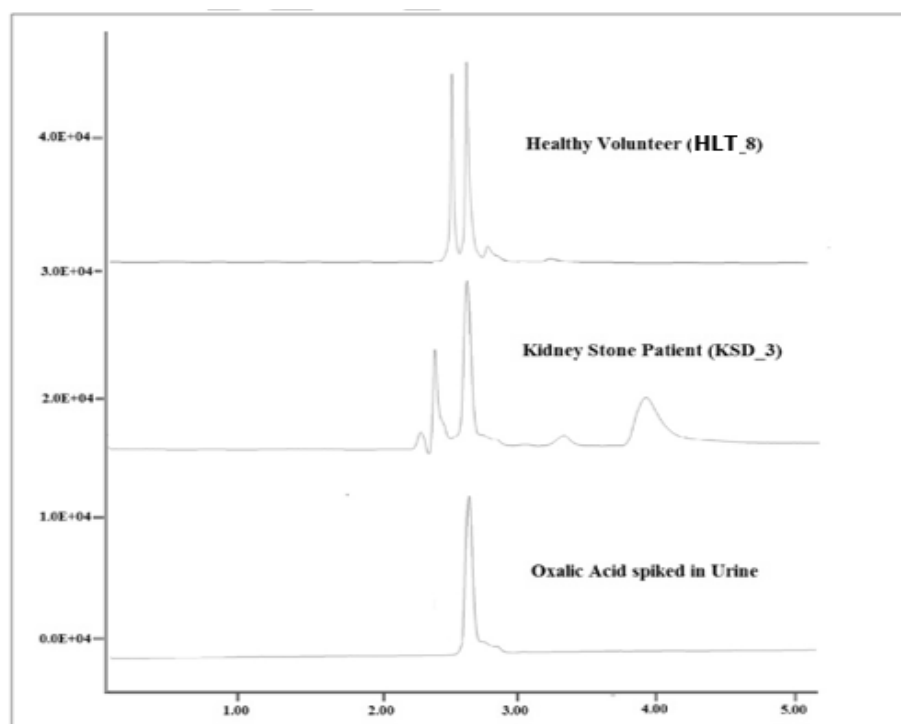


Fig. 1: Chromatograms of oxalic acid in various urine samples

Validation of chromatographic method

The validation of developed method was carried out by USFDA guidelines for bioanalytical method validation [33, 34]. The validation of method was carried out by performing following studies.

Selectivity

Individual specificity, in relation to endogenous urine components, was demonstrated by analysis of series of randomly selected oxalic acid-free urine samples. Typical chromatograms obtained after analysis of oxalic acid-free urine and urine samples after addition of oxalic acid are reported in fig. 2.

The retention time for the investigated oxalic acid was found to be different than that of endogenous urine components. Thus, it indicates selectivity of a method for elution of oxalic acid in urine.

Linearity and range study

The response factors calculated were found to be proportional to concentrations of analytes over the ranges tested as specified in table 1. This study was carried out separately on three consecutive days. The calibration curves were fitted by method of least square. The results of linearity study by regression analysis are given in table 1.

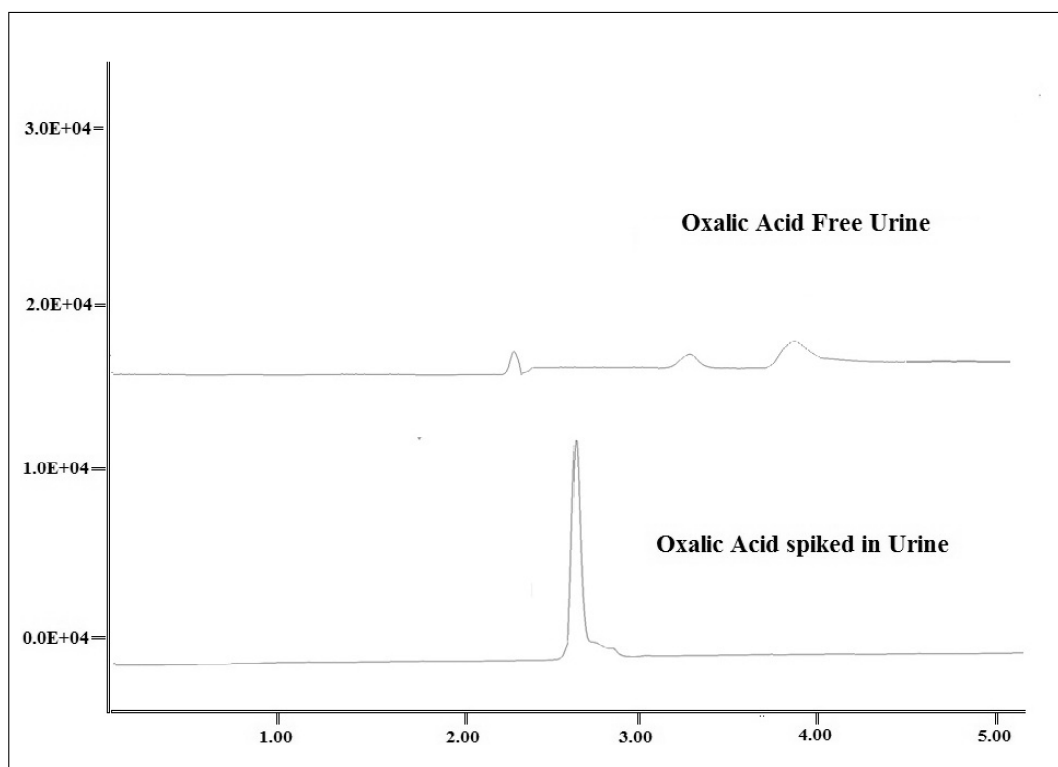


Fig. 2: Selectivity study of method by analyzing oxalic acid-free urine and oxalic acid spiked in urine

Lower limit of quantitation (LLOQ)

It is the lowest serum concentration of oxalic acid quantified with a coefficient of variation of less than 20%. The LLOQ value of oxalic acid was found to be 49 ng/ml.

Accuracy study

The recovery study has been carried out by analyzing quality control samples, (five determinations per three concentrations) spiked with the analyte. Results of accuracy study are given in table 3.

Table 3: Result of accuracy and precision studies

Analyte	Precision		Amount of pure oxalic acid added	% Concentration estimated mean ^a ±SD ^b
Oxalate	Intra-day	T1	5 mg	89.44±2.6215
		T2	10 mg	92.46±4.1412
		T3	15 mg	91.56±3.0555
	Inter-day	D1	-	90.57±3.0648
		D2	-	90.25±1.1468
		D3	-	90.28±1.4562

^aAverage of fifteen determinations (calculated at three levels 3×5; ^bStandard deviation, T-time, D-Day

Extraction recovery studies

The recovery represents the efficiency of an analytical method within the variation limit. The recovery in an assay is the detector response obtained from an amount of analyte added and recovered from the biological matrix (urine). Recovery experiments performed

in quadruplicate for an analyte by comparing the analytical results for extracted samples at three concentrations (equivalent to LLOQ, MQC, and HQC) with three unextracted concentrations that represent 100% recovery.

Results of the extraction recovery for analyses are given in table 1.

MQC-medium quality control concentrations, 50% of largest concentrations of calibration curve (linearity study)

HQC-high quality control concentrations, 75–90% of largest concentrations of calibration curve (linearity study)

% recovery = (Mean response of extracted samples/Mean response of un-extracted samples) × 100 [35].

Precision study

The precision study was carried out by analyzing the urine samples of three concentrations specified in the inaccuracy study for three consecutive days at two different times. This analysis illustrates the intraday and interday precision study. The urine samples were stored in between analysis in a freezer at -17 °C. The results of the precision studies are reported in table 3.

Stability study

This study was carried out by performing analysis of stock solutions, unextracted urine samples, and freshly prepared solutions at various atmospheric conditions and times as shown in the following headings as per guidelines as the mean of 6 readings with its standard deviation [36, 37].

Stock solution stability

To test the stock solution stability of oxalic acid, five aliquots of standard stock solutions were left at -17 °C for 3 d. Then, the concentrations were analyzed and compared with the fresh stock solution. The percentage recovery of oxalic acid was found to be 99.73±1.97 (mean±standard deviation).

Freeze and thaw stability

The effect of freeze and thaw cycles on the stability of urine samples containing analytes were determined by subjecting five aliquots of quality control (QC) samples at low, middle, and high concentration unextracted quality control samples to four freeze–thaw cycles. After completion of every cycle, the samples were analyzed, and the experimental concentrations were compared with the nominal values obtained by analyzing fresh samples. The accuracy values of three concentrations in two freeze–thaw cycles were calculated. The percentage recovery of oxalic acid was found to be 99.46±3.57 (mean±standard deviation).

Short-Term Stability-Five aliquots of QC samples at low, mid, and high concentration unextracted QC samples were kept at ambient temperature (15 °C) for 12 h in order to determine the short-term stability of analytes in urine. Then, the samples were processed and analyzed and the concentrations obtained were compared with the nominal values obtained by analyzing fresh samples. The percentage recovery of oxalic acid was found to be 100.23±2.25 (mean±standard deviation).

Post-preparation stability

In order to estimate the stability of oxalic acid in the prepared samples, five aliquots of QC samples at low, mid, and high concentration were kept at 4 °C for about 4 h. Then, the samples were analyzed and the concentrations obtained were compared with the nominal values obtained by analyzing fresh samples. The percentage recovery of oxalic acid was found to be 100.16±3.98 (mean±standard deviation).

CONCLUSION

The developed method was found to be accurate, precise, sensitive and selective for quantification of oxalate from the urine of kidney stone diseased patient and healthy volunteers. The method did not require pre-analysis sample processing as acetic acid is part of the mobile phase, used to concentrate oxalate in biological matrices. This method will be useful for estimation of oxalate from other biological matrices with few optimizations. The oxalate content data generated in this communication (table 2) was used for characterizing the gut microbial diversity of recurrent stone patients and healthy male subjects in comparatives by means diazotrophic bacterial colonization pattern and oxalate content in urine within into it.

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CONFLICTS OF INTERESTS

Declared none

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