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Rapid Ultra High Performance Liquid Chromatography–Tandem Mass Spectrometry Method for Quantification of Ethacrynic Acidin Human Plasma

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Abstract:*A sensitive and high throughput high performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) method has been developed for the determination of ethacrynic in human plasma. Sample preparation involved liquid–liquid extraction for ethacrynic and ethacrynic D5 as the internal standard (IS) in ethyl acetate from 200 µL human plasma. The chromatographic separation is achieved on a shimadzu HPLC and Gemini-NX 5µ C18 110 A° (50 X 4.6, mm) analytical column using an isocratic mobile phase, consisting of 0.1 % formic acid in water–acetonitrile (30:70, v/v). Detection was carried out on a tandem mass spectrometer inmultiple reaction monitoring mode using a negative electro pressure ionization interface.The method has been validated in linear range of 4.997-1000.283 ng/mL. The method is rugged and rapid with a total run time of 3.0 min and is applied to a bioequivalence study of 25 mg tablet formulation in 48 healthy Indian male subjects under fasting condition.*

Keywords: *Ethacrynic Acid, HPLC–MS/MS, Electrospray ionization, Bioequivalence, Human plasma*

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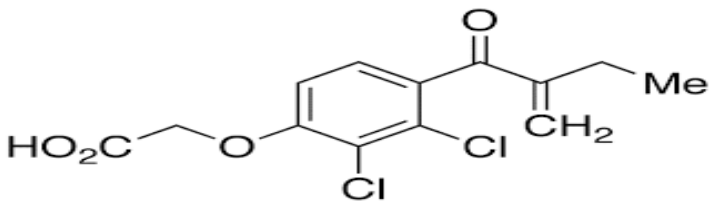
I. Introduction

Ethacrynic acid (Fig. 1) is a high-ceiling loop diuretic, primarily used in the treatment of pulmonary oedema when a rapid and potent diuretic action is required [1]. Ethacrynic acid is an unsaturated ketone derivative of phenoxy acetic acid chemically different from other known diuretic agents [2]. Diuretics are a wide class of drugs eliciting an increased production of urine. Apart from the prophylaxis of renal failure, their main indication in the clinical practice is for the mobilization of edemas and for the therapy of hypertension and of congestive heart failure [3]. Ethacrynic acid inhibits symport of sodium, potassium, and chloride primarily in the ascending limb of henle, but also in the proximal and distal tubules. This pharmacological action results in excretion of these ions, increased urinary output, and reduction in extracellular fluid**.**Some of analytical method using high performance liquid chromatography (HPLC) with UV detection [4-6], HPLC with diode-array UV detector [7], Simultaneous methods of HPLC and gas chromatographic-mass spectrometric (GC-MS) [8-10], gas chromatography method in guinea pig plasma [11], Simultaneous methods of HPLC-MS/MS and UPLC-MS/MS [12], Simultaneous methods of HPLC and LC-MS/MS [13], Simultaneous screening methods of LC-MS/MS [14-20] have been previously described for quantification in human plasma, urine or corneal tissues. These HPLC methods had runtimes that were too long (> 25 min) for analysis of large numbers of samples and also lacked specificity and ruggedness. A lower LLOQ and shorter runtime was still needed for analysis of clinical pharmacokinetic trial samples. The reported HPLC methods did not meet the needs of a clinical pharmacokinetic trial. The use of Liquid Chromatography (LC) coupled with electrospray tandem mass spectrometry has become the very popular technique in bioavailability studies due to the fast, sensitive, and reliable results generated by its use [21].

Ethacrynic acid



Ethacrynic acid D5

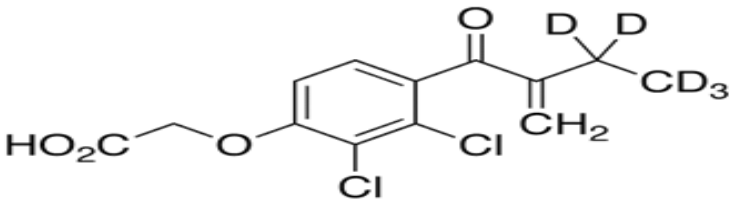


Figure 1. Chemical structure of Ethacrynic acid and Ethacrynic acid D5.

No LC method coupled with tandem MS (Mass Spectrometer) for human specimens has been published. In this research, we have present a new analytical method using only 0.200 mL of plasma volume is described with a lower limit of detection of 4.997 ng/mL, based on liquid-liquid extraction and LC-electrospray ionization (ESI)-MS-MS for the identification and quantification of ethacrynic acid. The small sample volume, short chromatography finished within 3 min has made the method adequate for pharmacokinetic evaluation. Moreover, the analyte is to be compared with deuterated internal standard, which is most useful in selectivity and matrix effect experiments by using LC-MS/MS. The objective of this study is to develop and validate a high-throughput HPLC-MS/MS method for reliable routine measurement of ethacrynic acid in human plasma to support clinical investigations. We have developed and validated method for performance of selective determination of ethacrynic acid contains combination of liquid-liquid extraction, reversed phase LC and tandem mass detection. This validated method was further evaluated in pharmacokinetic study of ethacrynic acid in 48 healthy human volunteers for single oral dose of a 25 mg immediate release formulation.

II. Exerimentals

1. Chemicals and reagents

Ethacrynic acid and ethacrynic acid D5 working standards were purchased from Clearsynth Research Center (Mumbai, India). High purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore (Bangalore, India). HPLC acetonitrile were purchased from Merck (Mumbai, India). Formic acid was purchased from Merck (Germany). Other reagents were used by analytical grade. Drug-free (blank) human plasma was obtained from Synchron Research Services Pvt.Ltd (Ahmedabad, India) and was stored at -70°C prior to use.

2. Preparation of calibration curve (CC) standards and quality control (QC) samples

A stock solution of ethacrynic acid (1 mg/mL) and internal standard (0.5 mg/mL) were prepared in methanol. Working standard solutions for calibration and quality controls were prepared from secondary standard solution by dilution with water: methanol (1:1, v/v). The stock solutions were stored at -70°C; intermediate stock and working stock solutions were stored at 2-8 °C until use. Calibration standards were prepared by spiking blank plasma with ethacrynic acid to get the final concentration of 4.997, 9.994, 49.969, 150.057, 500.192, 749.912, 900.255 and 1000.283 ng/mL. Quality control samples were prepared by spiking blank plasma with 800.227 ng/mL (HQC, high quality control), 496.191 ng/mL (MQC-01, medium quality control), 124.035 ng/mL (MQC-02, medium quality control), 13.644 ng/mL (LQC, low quality control) and 5.185 ng/mL (LLOQ QC, lower limit of quantification quality control) of ethacrynic Acid. The stability studies for quality control samples (HQC and LQC) spiked were stored at -70 ºC.

3. Sample Preparation

50 µL of the internal standard solution (501.242 ng/mL) and 200 µL of plasma sample were transferred into polypropylene tubes and vortexed briefly. 50 µL of concentrated hydrochloric acid were added into each tube and vortexed, followed by 3.0 mL of the extraction solvent ethyl acetate and vortexing for 10 min on rotospin at 50 RPM. The samples were centrifuged at 4000 rpm for 10 min at 10 °C. The supernatant from each sample was transferred into labeled polypropylene tubes and evaporated under nitrogen gas at 50 °C. The dried sample from each tube was reconstituted with 300 µl of mobile phase with vortexing. Finally, each sample was transferred into auto sampler vials and injected 10µL of sample into the liquid chromatographic system for analysis.

4. LC-MS/MS Method

LC/ESI-MS/MS was performed using a Shimadzu LCMS-8040 triple quadrupole mass spectrometer connected to a Shimadzu UPLC^{TM} system (Nexera-X2 SIL-30AC) chromatograph. Separation was performed on a reversed-phase Reprosil Gold XBD C18 column (100mm×3 mm , i.d., 3 µm) by using a mixture of 70 volumes of acetonitrile and 30 volumes of 0.1 % Formic Acid in water solution as mobile phase with isocratic elution at 0.700 mL/min. The column temperature maintained at 40˚C and auto-sampler temperature was maintained at 10˚C. The total run time for an LC-MS/MS analysis was 3.0 min and injection volume was 10µL. Mass spectrometric detection was performed using a shimadzu LCMS-8040 triple quadrupole mass spectrometer with ESI in the negative ion mode The MS conditions were as follows: interface voltage: 3.50 kV, Q1 pre-rod bias voltage: 23 V, Q3 pre-rod bias voltage: 23 V, collision energy: 11 eV (KPA), nebulizer gas flow rate: 3 L/min, drying gas flow rate:15 L/min, desolvation line temperature: 250°C, heat block temperature: 400°C and collision gas: 230 kPa. Lab solutions software (version 6.70, Shimadzu) was used for the system control and data processing.

5. Validation

The method has been validated for selectivity, sensitivity, linearity, precision, accuracy, matrix effect, carryover effect, reinjection reproducibility, recovery, partial volume, dilution integrity and stability following the US Food and Drug Administration (FDA) guidelines [22].The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [22]. Each calibration curve contained a single set of calibration standards and six replicates of QC at five concentration level.Selectivity was assessed, by comparing the chromatograms of eight different batches of blank plasma obtained from eight different sources (or donors) with those of corresponding standard plasma samples spiked with ethacrynic acid and ethacrynic acid D5. Sensitivity was determined by analyzing eight replicates of blank human plasma & plasma spiked with lowest level of the calibration curve.For the determining of intra-day accuracy & precision a replicates (n=6) analysis of plasma samples was performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ QC, LQC, MQC-02, MQC-01 and HQC samples. The inter-day accuracy & precision were assessed by analysis of three batches on different days. The precision was expressed as the relative standard deviation (RSD %) and the accuracy as the relative error (RE %). The %RSD as well as %RE expected to be within ±15% except for LLOQ, for which it should be within ±20%.

The recovery of ethacrynic acid and IS were determined by comparing the responses of the analytes extracted from replicates QC samples (n = 6) with the response of analytes from post extracted plasma sample at equivalent concentrations [23]. Recovery was determined at low (LQC), mid (MQC-01) and high (HQC) quality control concentrations, whereas the recovery of the IS was determined at a single concentration of 501.242 ng/mL. The matrix of plasma constituents over the ionization of analytes and IS was determined by comparing the responses of the post-extracted plasma standard QC samples (n = 6) with the response of analytes from neat samples at equivalent concentrations [23-24]. Matrix effect was determined at two levels (LQC and HQC) with eight different source of plasma for ethacrynic acid and IS (at 501.242 ng/mL). Dilution integrity was performed to extend the upper concentration limit with acceptable precision & accuracy. Five replicates each at a concentration of double the uppermost calibration standard (2000.566 ng/mL) were diluted (2 fold and 5 fold) with blank plasma and analyzed.All stability results were evaluated by measuring the area response (ethacrynic acid/IS) of stability samples against freshly prepared comparison standard at LQC and HQC levels. Auto sampler stability (wet extract), bench top (at room temperature) and freeze thaw stability were performed at LQC and HQC using six replicates at each level. Freeze thaw stability was evaluated by successive five cycles of freezing (at -20 and -70ºC) and thawing (without warming) at room temperature. Long–term stability of spiked plasma samples stored at -20 and -70ºC was also studied at both these levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15.0%.Process sample stability was evaluated with freshly spiked calibration curve and quality control samples and those freshly spiked quality control samples compared with re-injecting the same sample which (stability samples) were stored at 10ºC for 99h and at 25±5ºC for 7h.

Bench top stability was evaluated for 8h at room temperature and compared with freshly spiked plasma samples. All stability evaluations were based on back calculated concentrations.

6. Pharmacokinetic study

The method was applied to determine the plasma concentrations of ethacrynic acid from a bioequivalence in which 48 healthy male volunteers received one tablet (containing 25mg ethacrynic acid). The ethics committee approved the protocol and the volunteers provided with informed written consent to participate in the study according to the principles of the Declaration of Helsinki. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA [25]. Blood samples were obtained following oral administration of 25mg of ethacrynic acid into K_3EDTA vacutainer solution as an anticoagulant at pre-dose, 0.25h, 0.50h, 0.75h, 1.00h, 1.25h, 1.50h, 1.75h, 2.00h, 2.33h, 2.67h, 3.00h, 3.50h, 4.00h, 4.50h, 5.00h, 6.00h, 8.00h, 10.00h, 12.00h, and 24.00h post-dost. Plasma was harvested by centrifuging the blood using eppendorf centrifuge 5810R (Eppendorf, Germany) at 4000 ± 100 rpm for 5 minutes at 5 ± 2°C and stored frozen at −70±10˚C until analysis. An aliquot of 200µL of thawed plasma samples were spiked with IS and processed. Plasma concentration–time data of ethacrynic acid was analyzed by non-compartmental method using Kinetica Version 4.4 (Thermo USA). Regarding AUC_{0-t}, $AUC_{0-\infty}$ and C_{max}, bioequivalence was assessed by means of an analysis of variance (ANOVA) and calculating the standard 90% confidence intervals (90% CIs) of the ratios test/reference (logarithmically transformed data).

III. Result and Discussion

1. Method development

During method development, different options were evaluated to optimize detection parameters, chromatography and sample extraction.

2. Chromatography

The goal of this work was to develop and validate a simple, rapid and sensitive assay method for the quantitative determination of fat soluble analyte from plasma samples. LC-MS/MS has been used as one of the most powerful analytical tool in clinical pharmacokinetics for its selectivity, sensitivity, reproducibility and rapid analysis. Chromatographic conditions especially the composition of mobile phase, were optimized through several trials to achieve good resolution and increase the signal of analytes, as well as short run time. The resolution of peaks was achieved with acetonitrile and 0.1 % formic acid in water (70:30, v/v) with a flow rate of 0.700 mL/min, on a Gemini-NX 5µ C18 110 A° (50 X 4.6, mm) column and was found to be suitable for the determination of electrospray response for ethacrynic acid and IS. Under these conditions the chromatographic analysis of standard solution containing the present analytes showed a good separation of ethacrynic acid with retention time of 1.96 min and IS with retention tine of 1.97 min, making the run time only 3.00 min and this met the requirement for a high sample throughput.

3. Mass Spectra

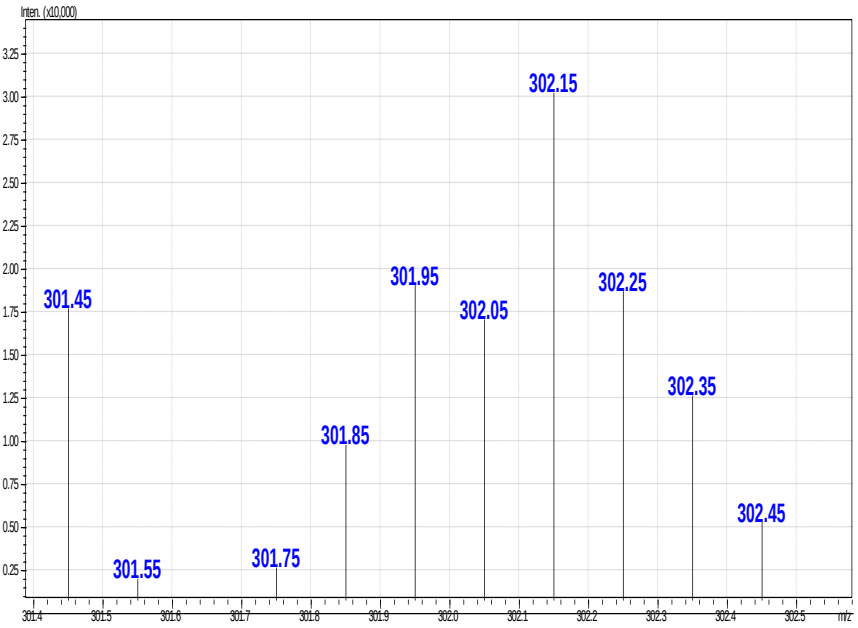
LC/MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. In order to optimize MS conditions, they were performed using both positive and negative ionization modes for ethacrynic acid and ethacrynic acid D5 (IS) into the ESI source of the mass spectrometer and parameters such as capillary (ESI). However, with best tuning of the MS parameters, the consistent response found was much higher in negative ionization mode. Nebulizer and dessolvation gases were optimized to obtain better spray shape resulting in better ionization and droplet drying to form the deprotonated ionic ethacrynic acid and IS molecules. The full scan spectrum was dominated by deprotonated molecules [M-H]- m/z 302.15 and 307.25 for ethacrynic acid and ethacrynic acid D5. The mass spectra of precursor ions of ethacrynic acid and ethacrynic acid D5 are presented in Fig. 2 with most intense peak. And major fragment ions observed in each product spectrum were at m/z 244.05, 249.00 respectively for ethacrynic acid and IS. The selected fragments of each compound, as product ions to be monitored, are indicated in Fig. 3 with most intense peak.

4. Specificity and selectivity

Eight different lots of plasma along with one lipemic plasma and one haemolysed, plasma were analyzed to ensure that no endogenous interferences were present at the retention time of ethacrynic acid and IS. Eight LLOQ level samples along with plasma blank from the respective plasma lots were prepared and analyzed. In all plasma blanks, the response at the retention time of ethacrynic acid was less than 20% of LLOQ response and at the retention time of IS, the response was less than 5% of mean IS response in LLOQ.

As shown in Fig.4 no interference peaks from endogenous compound is observed at the retention times of analyte and IS**.**

(a)



(b)

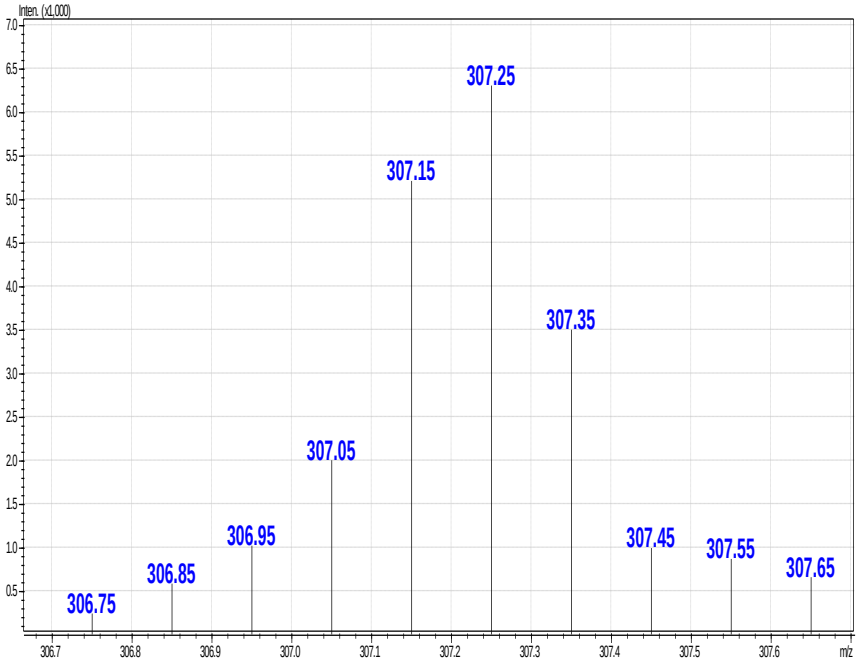
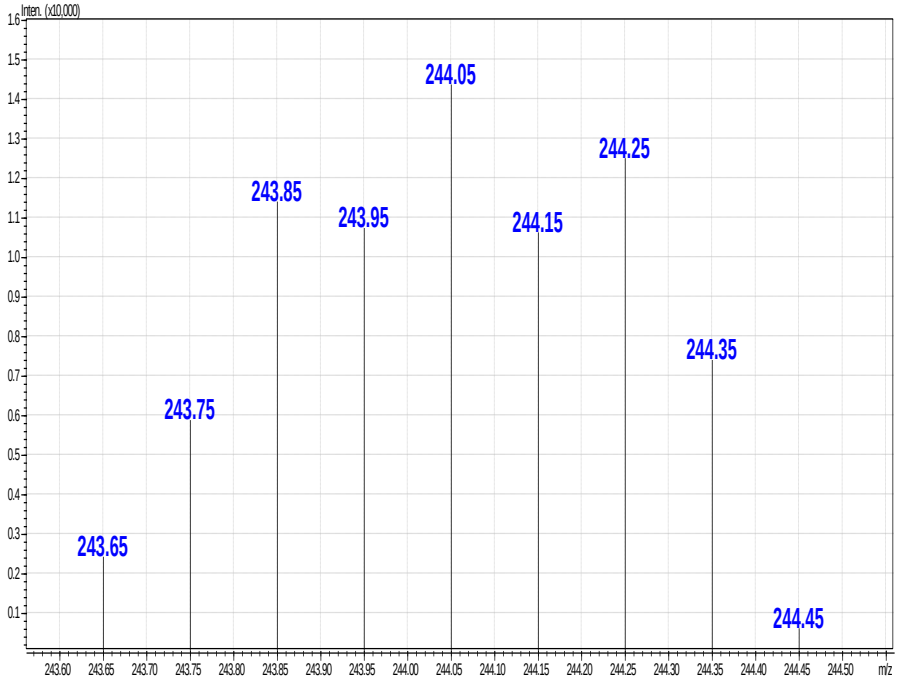


Figure. 2. Electrospray negative ion mass spectra of the precursor ion of (a) ethacrynic acid and (b) ethacrynic acid D5.

5. Linearity

`The plasma calibration curve was constructed using calibration standards 4.997- 1000.283 ng/mL for ethacrynic acid. Linearity was assessed by a weighted ($1/c^2$) least squares regression analysis. The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/c^2$ weighing factor, giving a mean linear regression equation for the calibration curve of: y = mx + c for ethacrynic acid where y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.99 or better; Table 1 summarizes the calibration curve results.

(a)



(b)

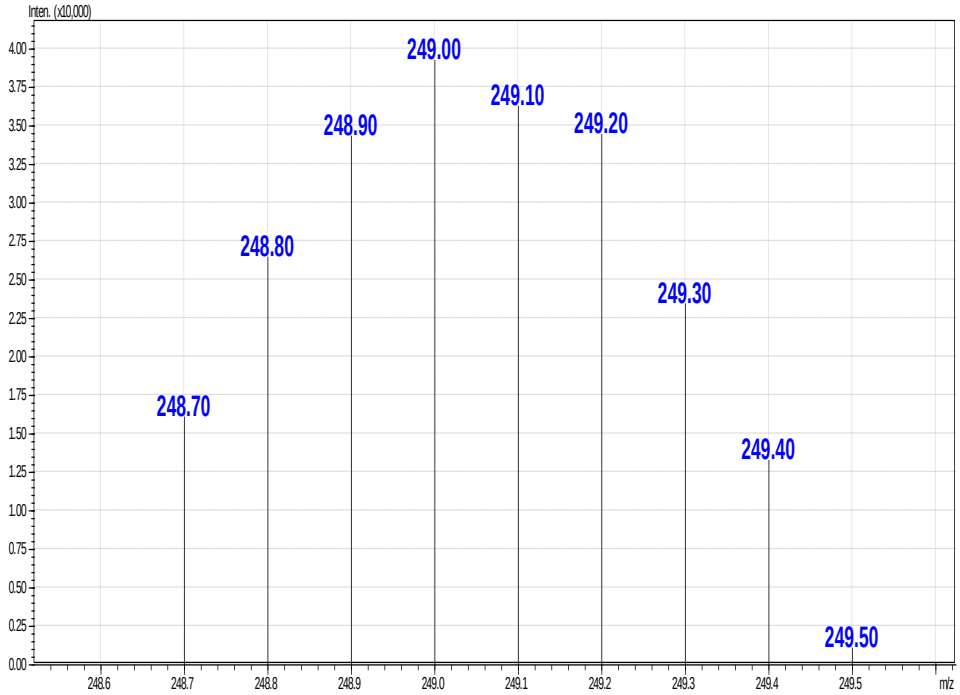


Figure 3. Electrospray product ion mass spectra for (a) ethacrynic acid and (b) ethacrynic acid D5.

To determine weighting factor for ethacrynic acid calibration standards accuracy results were calculated for three P&A batches with linear regression (y=mx+c) using weighting factors as 1/c, $1/c^2$ and no weighting. The percent relative error (%RE) were calculated for each standards level as percent difference between the observed value of all %RE are summed for each weighting factor in three calibration standards. The weighting factor that gives the smallest values of the sum of %RE is taken as concentration response relationship for ethacrynic acid. In our case, it was $1/c^2$.The ε%RE for weighting factor none, 1/C and 1/C2 was 261.6%, 59.3 % and 53.8 %, respectively for ethacrynic acid.

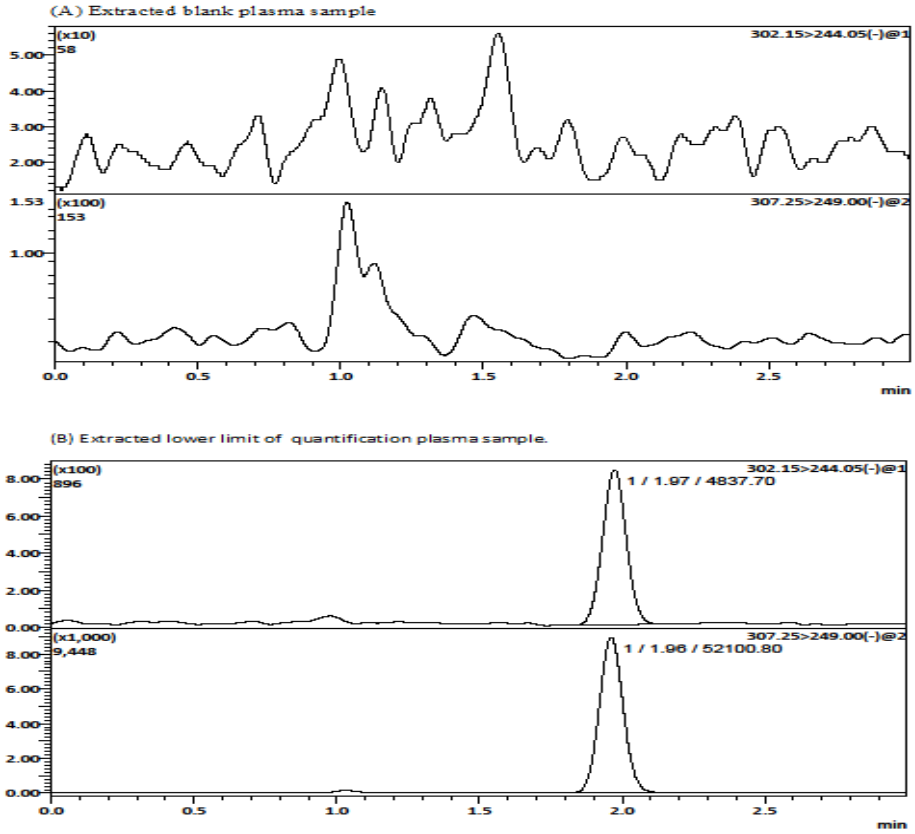


Figure 4. Representative chromatograms of (A) Extracted blank plasma sample; (B) Extracted lower limit of quantification plasma sample.

Analyte	Concentration added (ng/mL)	Concentration found (mean ; ng/mL)	Precision (%)	RE (%)
Ethacrynic Acid	4.997	4.947	2.00	-1.00
	9.994	10.264	4.00	2.70
	49.969	48.179	0.40	-3.58
	150.057	149.964	0.80	-0.06
	500.192	506.028	3.50	1.17
	749.912	740.225	1.60	-1.29
	900.255	921.083	2.30	2.31
	1000.283	997.884	2.00	0.24

Table 1. Precision and accuracy data of back-calculated concentration of calibration samples for ethacrynic acid in human plasma.

6. Sensitivity

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision, and it was found to be 4.997 ng/mL for ethacrynic acid. The intra-day precision at the LLOQ was 1.2%, and the intra-day accuracy was 4.7% for ethacrynic acid respectively (Table 2).

	Intra-day precision(n=6)			**Inter-day precision (n=18)**		
Concentration added (ng/mL)	Concentration found (mean ; ng/mL)	Precision (%)	RE (%)	Concentration found (mean ; ng/mL)	Precision (%)	RE (%)
800.385	840.507	1.3	5.0	851.043	1.7	6.3
496.239	512.764	1.4	3.3	522.433	3.6	5.3
124.060	127.287	0.9	2.6	121.865	3.4	-1.8
13.647	14.261	1.5	4.5	14.572	2.3	6.8
5.049	4.887	1.2	-3.2	4.902	4.7	-2.9

Table 2. Precision and accuracy of the method for determining ethacrynic acid concentration in plasma samples.

7. Precision and accuracy

The results for intra-day and inter-day precision and accuracy for ethacrynic acid in plasma quality control samples are summarized in Table 2. The intra-day precision and accuracy was ≤1.5% and ≤5.0% for ethacrynic acid respectively. The inter-day precision and accuracy was ≤4.7% and ≤6.8% for ethacrynic acid respectively.

8. Recovery

Six aqueous replicates (samples spiked in mobile phase) at low (LQC), middle (MQC-01) and high (HQC) quality control concentration levels for ethacrynic acid were prepared for recovery determination and the areas obtained were compared versus the areas obtained for extracted samples of the same concentration levels from a precision and accuracy batch run on the same day.The mean recovery was 78.88% and 87.97% for ethacrynic acid and ethacrynic acid D5, respectively. The observed variability in % CV between these levels was 6.5% for ethacrynic acid and 7.0 % for IS. The recovery obtained for analyte was higher than that of other reported methods.

9. Matrix Effect

The matrix effect was evaluated by analyzing QC samples. Average matrix factor values (matrix factor = response of post-spiked concentrations/response of neat concentrations) obtained were +0.92 (CV 2.2%, n= 8) at LQC and +0.88 (CV 1.1%, n = 8) at HQC level for ethacrynic acid, whereas on IS it was found to be +0.95 (CV 3.2%, n= 8) at tested concentration of 501.242 ng/mL. Matrix effect was not observed at analyte and IS retention time.

10. Dilution integrity

The upper concentration limits can be extended to 2000.567 ng/mL for ethacrynic acid by a 2-fold or 5-fold dilution with human plasma. The nominal concentration (%) for 1/2 and 1/5 dilution samples ranged from 500.141 and 200.056 ng/mL, respectively. The precision (coefficient of variation) for 1/2 and 1/5 dilution samples of ethacrynic acid were 1.5% and 1.2%, respectively, while accuracy were for 1/2 and 1/5 dilution samples of ethacrynic acid were 99.0% and 96.1%, respectively.

11. Stability and Incurred sample reanalysis

The stability of the analyte in human plasma under different temperature and timing conditions was evaluated and are enumerated in Table 3. QC samples were subjected to long-term storage conditions (-70°C), and to freeze–thaw stability studies. All the stability studies were conducted at two concentration levels (13.647 and 800.385 ng/mL for ethacrynic acid as low and high QC values) with five determinations for each. For process stability, the results indicated that the difference in the back-calculated concentration from time 0 to 99 h is 1.2% for ethacrynic acid respectively which allowed us to conclude that processed samples are stable at least for 99 h at 10°C in the auto-sampler. For bench top stability, the results allowed us to conclude that both analytes are stable for at least 8 h at room temperature in plasma samples. Freeze and thaw stability results indicated that the repeated freeze and thawing (five cycles) did not affect the stability of ethacrynic acid. Long-term stability of the analytes in plasma at -20°C and -70°C was found to be stable for at least 77 days at -70°C.An incurred sample re-analysis (ISR) was also conducted by computerized random selection of subject samples, 10 % of total no (2016 samples) of subject samples, which is 201 samples were analyzed. The selection criteria included samples which were near the Cmax and the elimination phase which is having concentration at least 3 times of LLOQ in the pharmacokinetic profile of the drug. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the value should not be more than ±20 % [26]. The overall view of results is given residual graph (Fig. 5).

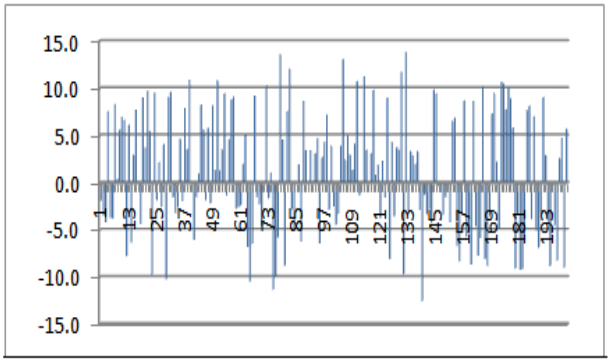


Figure. 5. Residual graph for Incurred sample reanalysis.

Stability	**n**	**Spiked Concentration (ng/mL)**	**Mean calculated comparison sample concentration (ng/mL)**	**Mean calculated stability sample concentration (ng/mL)**	**Mean percentage change**
Process/Wet extract [a]	6	13.647	14.787	14.666	-0.8
	6	800.385	835.853	845.844	1.2
Process/Wet extract [b]	6	13.647	14.862	15.015	1.0
	6	800.385	881.263	898.884	2.0
Bench top [c]	6	13.647	14.862	15.014	1.0
	6	800.385	881.263	894.749	1.5
Freeze and thaw [d]	6	13.647	14.147	14.089	-0.4
	6	800.385	805.678	805.547	0.1
Freeze and thaw [e]	6	13.647	14.147	14.089	-0.4
	6	800.385	805.678	818.308	1.6
Long-term [f]	6	13.647	13.996	13.892	-0.7
	6	800.385	799.831	794.332	-0.7
Long-term [g]	6	13.647	13.996	13.836	-1.1
	6	800.385	799.831	798.192	-0.2

[a] After 99 h in autosampler at 10°C; [b] After 7 h in room temperature (25±5ºC); [c] after 8 h at room temperature; [d] after five freeze and thaw cycles at -20°C; [e] after five freeze and thaw cycles at -70°C; [f] at -20°C and [g] at -70°C for 77 days.

Table 3. Stability samples result for ethacrynic acid.

12. Pharmacokinetic study

The proposed method was applied to determine ethacrynic acid levels in plasma for a bioequivalence study in 48 healthy Indian male volunteers who were orally administered 25 mg ethacrynic acid in tablet form. High-throughput sample analysis is of particular importance for studies that require the analysis of large numbers of samples, and the described liquid-liquid method of sample preparation is suitable for this purpose. Fig. 6shows the mean plasma concentration-time curves for the two formulations. Pharmacokinetic parameters derived from these curves are presented in Table 4. No significant differences were observed between the two formulations in terms of; C_{max}, AUC_{120h}, AUC_{inf}, T_{max}or $t_{1/2}$.

Pharmacokinetic parameters Ethacrynic Acid	Test A	Reference
Tmax (h)	1.80 ± 0.84	1.43 ± 0.35
C_{max} (ng/mL)	282.754 ±144.30	253.514±184.78
AUC_{0-36} (h·ng/mL)	366.790 ±124.11	352.937±111.66
AUC_{0-inf} (h·ng/mL)	372.909 ±126.00	372.027±127.02
$t_{1/2}$ (h)	10.998 ± 9.94	8.337 ± 3.64

Table 4. Bioequivalence parameters obtained after oral administration of reference drug and test drug at the ethacrynic acid dose of 25 mg.

To our knowledge, the present method is the first described for the analysis of ethacrynic acid in human biological samples by means of HPLC-MS-MS. We have developed and fully validated a highly sensitive, rapid and rugged HPLC-MS/MS method for the determination of ethacrynic acid in human plasma. Most of the analytical methods reported for quantification of ethacrynic acid in biological matrix was longer and lacks sensitivity. The basic underlying advantage of this optimized method is that it utilizes only 0.200 mL of plasma, a very simple liquid-liquid extraction procedure and short chromatographic run time of this assay are particularly suitable for routine assay. The method was selective in the presence of medications commonly used by human volunteers. Ion suppression/enhancement was studied by the post column infusion of analyte and post extraction spiking technique. The proposed method has been successfully applied to a bioequivalence study of 25 mg ethacrynic acid tablet formulation in 48 healthy males under fast condition. Reliability of the measurement of study samples is shown by incurred sample reanalysis.

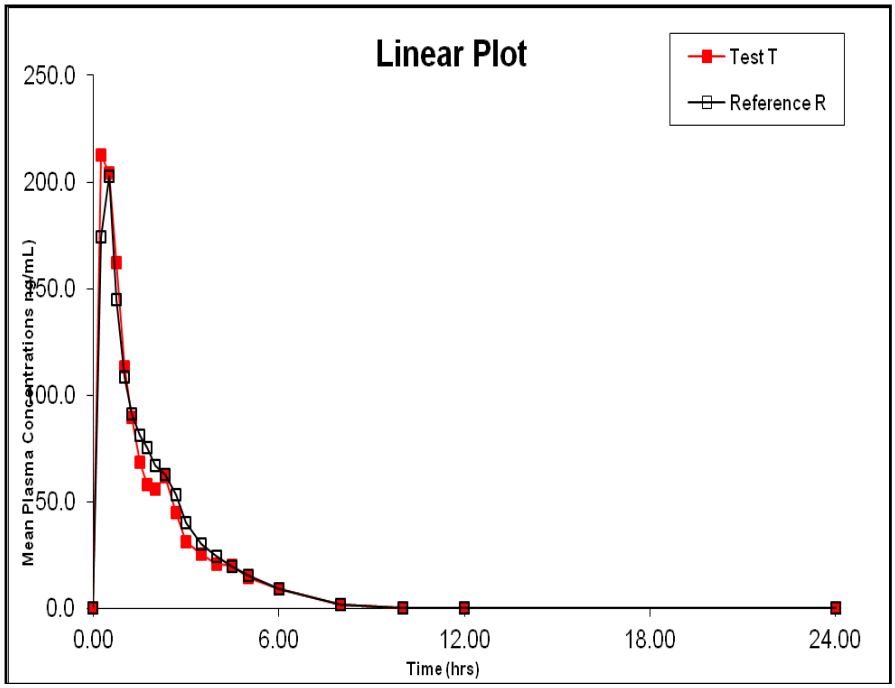


Figure. 6. Mean plasma concentration–time profile of ethacrynic acid in human plasma following oral dosing of 25mg ethacrynic acid tablet to 48 subjects.

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