# SIMULTANEOUS QUANTITATION OF ESTRADIOL AND ESTRONE IN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY WITH ELECTROSPRAY IONIZATION

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**ABSTRACT**

A simple, rapid, sensitive liquid chromatography-tandem mass spectrometry method was developed and validated for simultaneous analysis of endogenous estradiol and estrone in human plasma. The analytes were extracted by liquid-liquid extraction with dicholormethane, which was then evaporated and derivatized with dansyl chloride, and injected into the LC/MS/MS system. The chromatographic separation was performed on reverse phase sunfire C18 column with a mobile phase comprising formic acid and acetonitrile in gradient mode. The mass transitions of m/z 506.12/170.66 for 3-dansyl-estradiol, m/z 504.10/170.66 for 3-dansyl estrone were monitored using multiple reaction-monitoring (MRM) modes for quantification. With the optimum tuning parameters, the lower level of quantification for estradiol in human plasma was 15.64 pg $mL^{-1}$ and 16.0 pg $mL^{-1}$ for estrone and estradiol, respectively. Acceptable precision and accuracy were obtained for concentrations over the standard curve range of 15-1250 pg $mL^{-1}$. This procedure could potentially be used in the investigation of estrogen for the pathophysilogical assessment and other clinical applications, including pharmacokinetic studies.

**Keywords:** LC MS/MS, Estradiol, Estrone, Endogenous estrogen

## INTRODUCTION

Estrogens are the natural steroids mainly produced in the ovaries and testes, which influence the growth, development, differentiation and function of peripheral tissues of the female and male reproductive system. It has been known that estrogens play an important role in the maintenance of bone mineral density[1,2], cognitive[3-6] and cardiovascular functions[7-10]. They are also known to impact in developing breast, uterine, or colorectal cancer[11-16].

Estrone (E1) and estradiol (E2) are the two major metabolically active estrogens, which are synthesized from androgenic precursor ($\Delta^4$-androstenedione and testosterone) by demethylation and aromatization, which are transformed to each other by the action of 17 β-hydroxy-steroid dehydrogenase. E2 is the predominant bioactive estrogen in premenopausal, nonpreganant women, circulating at 1.5-4 times the concentration of E1, thus forming an integral part of the assessment of female reproductive function, including studies of infertility, oligo-amenorrhea, and menopausal status[17,18]. Also the assay of estrogen is very important in the clinical situations like inborn errors of sex-steroid metabolism, disorders of puberty, estrogen deficiency, breast cancer, male osteoporosis, alzheimer disease, and cardiovascular disorders[19-31].

In order to quantify plasma concentrations of estrogens in clinical pathophysiological diagnosis and assessments, it is necessary to develop and validate an assay with appropriate sensitivity, selectivity, accuracy and precision. Of the various analytical methods available for the analysis of

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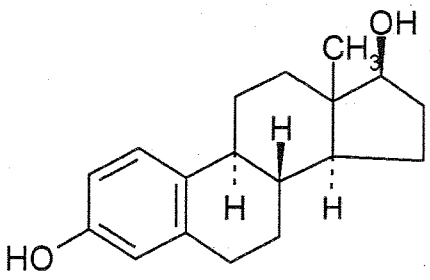
estrogen, high sensitivity E2 immunoassay, though useful but suffer from the drawback of handling radioactive materials and prolonged incubation[32,33]. E1 and E2 assays based on gas-chromatography-mass spectrometry (GC-MS) address many of the shortcomings of automated immunoassays and RIAs, but the run times may be longer than 30 min/sample, limiting throughput[34-37]. However, recently the application of liquid chromatography/mass spectrometry (LC-MS) and tandem mass spectrometry coupled to liquid chromatography (LC/MS/MS) have been shown to be superior in terms of both sensitivity and sample throughput and has thus replaced the immunoassays and various other cumbersome analytical methods[38,39]. Hence it is essential to establish an assay capable of quantifying estrogens at lower concentrations. At the same time, it is expected that this method would be efficient in analyzing large number of clinical samples obtained for pathophysiological assessment, pharmacokinetics studies after therapeutic doses of estrogens. In the present paper we report the LC-MS/MS method developed and validated for the simultaneous determination of E1 and E2 in human plasma.

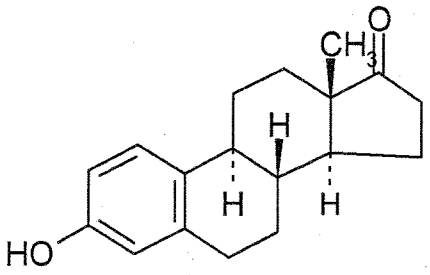
## MATERIALS AND METHODS

### Chemicals

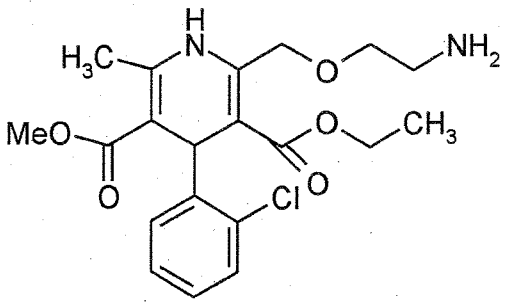
The pure substances of estradiol, estrone hemidhydrate were procured from the council of Europe, European Pharmacopoeia (Strasbourg, Cedex). Amlodipine besilyate used as internal standard was from Intas Pharmaceuticals (Ahmedabad, India). Chemical structures are presented in Fig. 1. Dansyl chloride was procured from Acros organics (New Jersey, USA). Bovine serum albumin was used as control (Sigma-Aldrich). HPLC grade acetonitrile, methanol were used for the chromatography (Rankem, India). All other chemicals used were of analytical grade. Water was deionized using a Milli-Q system from Millipore (Bedford, MA, USA).




Estradiol




Estrone




Amlodipine

**Fig. 1: Chemical structures of Estradiol, Estrone and Amlodipine**

### Instrumentation and Chromatographic Conditions

The HPLC Alliance HT 2795 series (Waters, USA) is equipped with binary pump, degasser and autosampler with thermostat, thermostated column compartment and control module. The chromatography was on Waters Sun fire C18 column (3.5 mcm, 21 mm x 50 mm i.d.). The mobile phase comprised of 0.1 % formic acid buffer and acetonitrile in gradient mode with a flow rate of 0.25 mL/min.

**Table I : Tandem mass-spectrometer main working parameters**

| Parameter | Value |
|---|---|
| Capillary voltage (kV) | 3.2 |
| Cone voltage (V) | 30 |
| Source temperature ($^0$) | 150 |
| Desolvation temperature ($^0$) | 400 |
| Cone gas flow (L/h) | 49 |
| Desolvation gas flow (L/h) | 500 |
| Extractor (V) | 5 |
| Dwell time (sec) | 0.2 |
| Mode of analysis | $ES^+$ |
| Ion transition for Estradiol (m/z) | 506.13/170.66 |
| Ion transition for Estrone (m/z) | 504.10/170.66 |
| Ion transition for amlodipine (m/z) | 409.19/238.24 |

Mass spectrometric detection was performed on ESI triple quadrupole instrument Quattro Premier (Micromass MS technologies, Waters, USA) using multiple reaction monitoring (MRM). The main working parameters of the mass spectrometer are summarized in Table I. Data processing was performed on QuanLynx 4.0 software package (Waters).

## Sample Processing

For method development and validation, blood samples from thirty-seven healthy postmenopausal women aged between 45-70 years were collected and centrifuged at 1500 g for 10 min. Plasma was separated and stored at -20$^0$ to assess the specimen type suitability and stability. A 500 mcL volume each of the plasma samples were transferred individually to a 15 mL glass test tube. Added 4 mL aliquot of dichloromethane into each tube using multipette plus (eppendorf, USA). The sample was vortex-mixed for 4 min using Multi-Pulse Vortexer (Glas-Col, Terre Haute, USA). The upper aqueous layer was removed and the remaining organic layer was transferred to a 5 mL glass tube and evaporated to dryness using Turbo Vap LV Evaporator (Zymark, Hopkinton, MA, USA) at 50$^0$ under a stream of nitrogen. Then the residue was dissolved in 200 mcL of $NaHCO_3$ (100 mM; pH 10.5). Added 200 mcL dansyl chloride solution (1mg/mL in acetone), vortexed and heated for 3 min at 60$^0$. Added 25 mcL of internal standard (1.13 mcg/mL), vortexed and 30 mcL aliquot was injected into chromatographic system.

## Method Validation

Validating a quantitative analytical method for an endogenous substance pose challenges for the analytical chemist, mainly due to the lack of control biological matrix that is free of the analyte and can be used for the preparation of spiked calibration standards and quality control samples. Assay accuracy measurements must take into account the endogenous basal analyte concentration. Several approaches have been published to date to accommodate this, including the use of stripped and substitute matrices, standard addition to pooled matrix, or use of stable isotope labelled standards. Representative examples of some of these approaches to endogenous analyte quantification can be found in the recent literature[40-42].

In developing the present assay, all available plasma matrices had detectable concentrations of either of E2 or E1, approaching the lower limit of quantification (LLOQ). Therefore, the calibrating standards for the assay were prepared in a substitute matrix solution containing 1 mg/mL bovine serum albumin in phosphate buffered saline. Aliquot of the stock solution of E2, E1 (1 mg/mL) was serially diluted in standard curve matrix to prepare 8-point calibration curve ranging from 15.64 - 1253.83 pg/mL for E2 and 16.09 - 1290.22 pg/mL for E1. The applicability of this substitute matrix to accurately and precisely quantify E2 and E1 in biological matrix was established by using four different concentrations of quality control (QC) samples of each of E2 and E1 across the calibration range.

The method was validated for specificity, precision and accuracy and for the recovery. Short term and long term stability of the drug in plasma

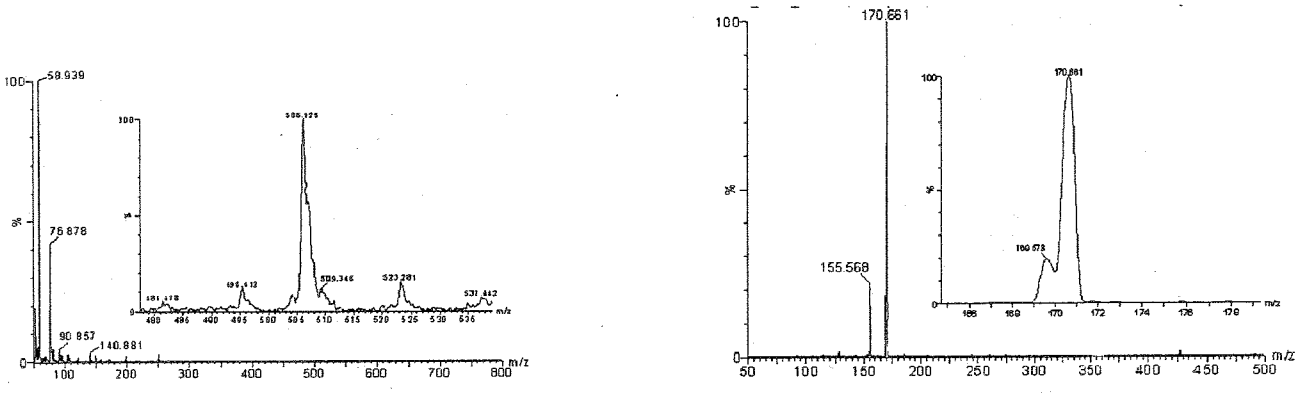


**Fig. 2: Full scan positive ion mass spectra and product ion of estradiol**

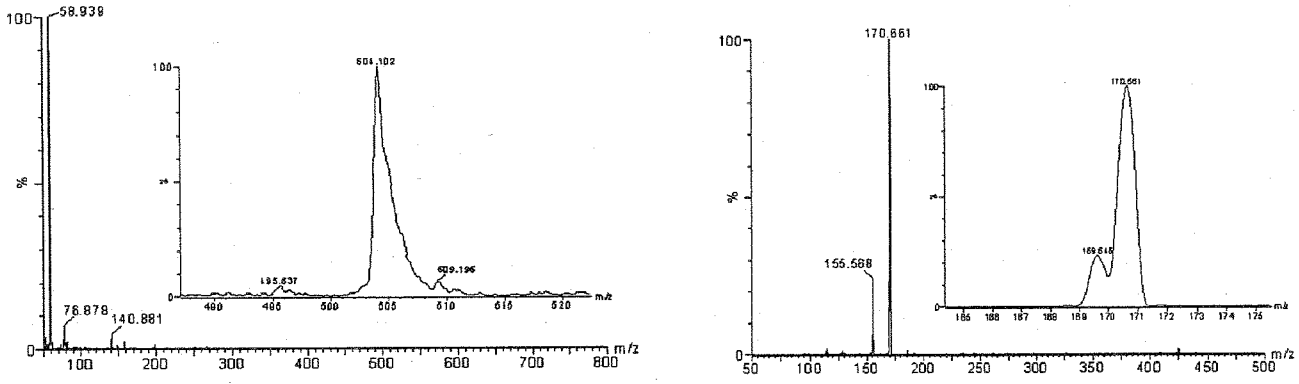


**Fig. 3: Full scan positive ion mass spectra and product ion of estrone**

also was evaluated by checking freeze thaw, auto sampler and bench top stability at two QC levels on the same sample matrix.

## RESULT AND DISCUSSION

In the present work LC-MS/MS was used to analyze estrogens, as it is beneficial in developing a selective and sensitive method in MRM mode. The positive ion mass spectrum and product ion mass spectrum of E2, E1 and the I.S are shown in Fig. 2, 3 and 4 respectively. $[M+H]^+$ was predominant ion in the parent spectrum and was used as the precursor ion to obtain product ion spectra. The most sensitive mass transition for E2 was from m/z 506.12 to 170.66, for E1 it was m/z 504.10 to 170.66 and m/z 409.12 to 238.23 for the I.S. Thus the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for the analyte and are given in Table I.

### Method Development

The functional sensitivity of the earlier LC-MS/MS multiple-reaction monitoring experiments corresponding to underivatized E1 (m/z 269/145) and E2 (m/z 271/145) was insufficient for our intended clinical applications, particularly with regard to E2, and therefore pursued derivatization to improve ionization efficiency and experimental sensitivity. Derivatization with dansyl chloride was highly effective and was therefore used for all studies described for the LC-MS/MS method. To cope with the matrix effect due to endogenous drug substances, bovine serum albumin in phosphate buffer was used as control in the validation studies. Different mobile phases consisting of water-methanol

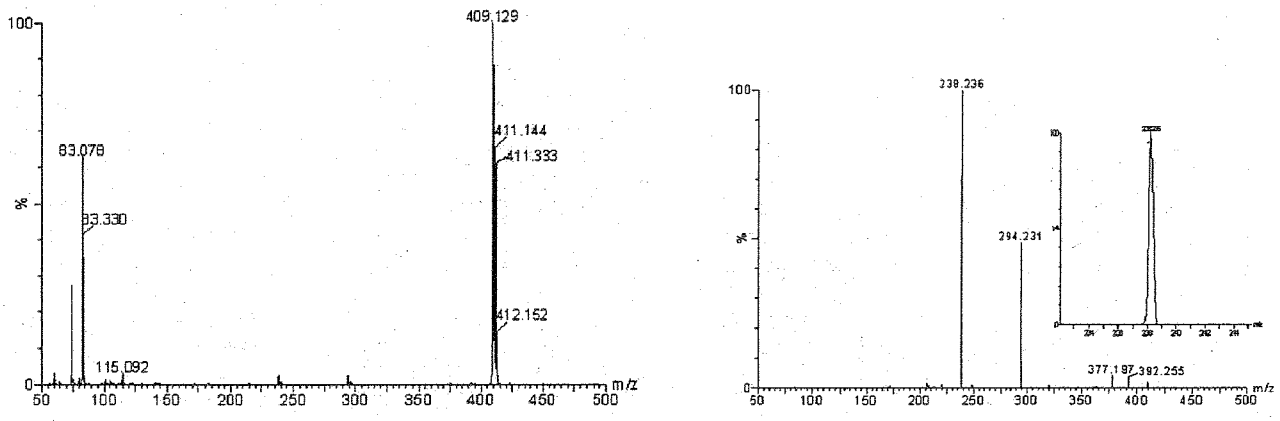



**Fig. 4: Full scan positive ion mass spectra and product ion of amlodipine (I.S)**

or water-acetonitrile were evaluated to improve HPLC separation and enhance sensitivity in MS. Modifiers such as formic acid and ammonium formate alone or in combination in different concentrations were added. The best signal was achieved using 0.1% formic acid buffer and acetonitrile in gradient mode. The formic acid was found to be necessary in order to lower the pH to protonate the E1 and E2 and thus deliver good peak shape. The percentage of formic acid was optimized to maintain this peak shape whilst being consistent with good ionization and fragmentation in the mass spectrometer.

The tandem mass spectrometer allows the selective detection of substances with varying masses or fragments without chromatographic separation. The development of the chromatographic system was focused on short retention times and co-elution of E2, E1 and I.S., paying attention to matrix effects as well as good peak shapes. A high proportion of organic solvent (0.1% formic acid/acetonitrile 10/90, v/v) was used to co-elute E2 and E1 at retention time of 5.22 and 5.01 min respectively. Flow rate of 0.25 mL/min produced a good peak shape and brought the runtime to 6 min.

## Specificity

LC-MS/MS analysis of the blank human plasma samples showed the presence of endogenous E2

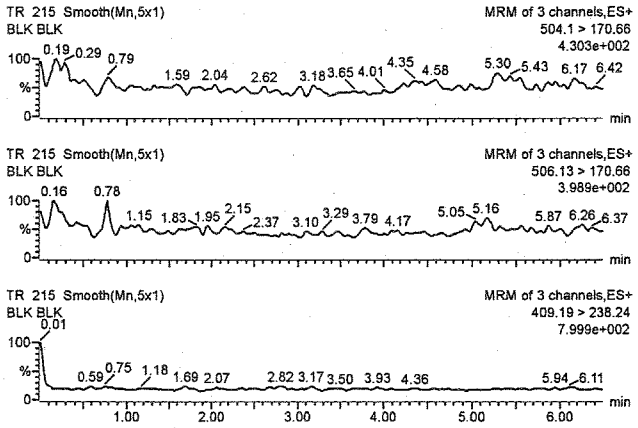



**Fig. 5: MRM ion-chromatograms showing the absence of interference in the substituted matrix**

and E1 and no interference with the I.S. Hence the specificity of the method was established by comparing with a substitute matrix comprising of bovine serum albumin. Representative chromatograms of E1 and E2 are shown in substituted matrix (Fig.5), blank plasma fortified with IS (Fig.6) and clinical plasma samples (Fig.7)

## Linearity

Calibration curve was linear over the concentration range of 15-1250 pg/mL for both the analyte. The eight-point calibration curve gave acceptable results and was used for all the calculations. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.995 and 0.996 for E2 and E1

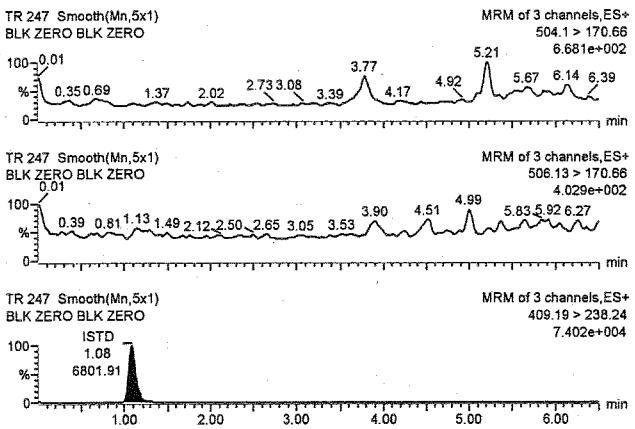



**Fig. 6: MRM ion-chromatograms of blank human plasma showing endogenous estrone, estradiol with the added IS**

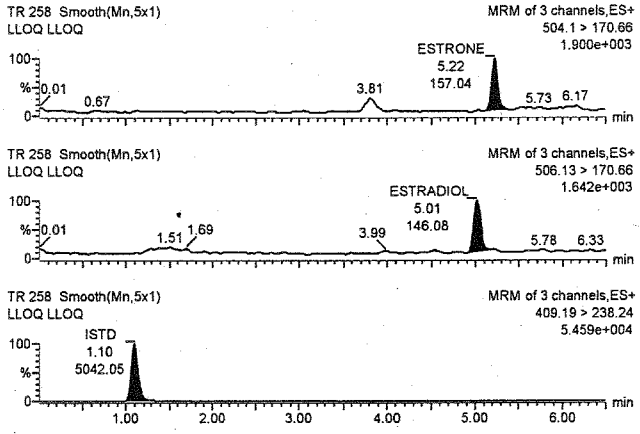



**Fig. 7: Representative MRM ion-chromatgrams of estrone, estradiol in the clinical samples along with the added internal standard**

**Table II : Precision and accuracy data of back-calculated concentrations of calibration samples for estradiol and estrone**

| Estradiol | | | | Estrone | | | |
|---|---|---|---|---|---|---|---|
| Concentration added (pg/mL) | Concentration found[a] (pg/mL) | Precision (%) | Accuracy (%) | Concentration added (pg/mL) | Concentration found[a] (pg/mL) | Precision (%) | Accuracy (%) |
| 15.64 | 15.68 ± 0.94 | 6.0 | 100.3 | 16.09 | 16.82 ± 0.54 | 3.2 | 104.6 |
| 31.28 | 31.34 ± 3.79 | 12.1 | 100.2 | 32.18 | 30.77 ± 2.98 | 9.7 | 95.6 |
| 62.56 | 60.75 ± 1.20 | 2.0 | 97.1 | 64.37 | 58.62 ± 4.32 | 7.4 | 91.1 |
| 189.58 | 195.38 ± 9.40 | 4.8 | 103.1 | 195.08 | 185.83 ± 7.8 | 4.2 | 95.3 |
| 631.93 | 684.95 ± 23.85 | 3.5 | 108.4 | 650.27 | 702.96 ± 39.47 | 5.6 | 108.1 |
| 902.76 | 878.78 ± 75.05 | 8.5 | 97.3 | 928.96 | 899.89 ± 55.05 | 6.1 | 96.9 |
| 1128.45 | 1140.66 ± 38.81 | 3.4 | 101.1 | 1161.20 | 1230.04 ± 35.31 | 2.9 | 105.9 |
| 1253.83 | 1160.13 ± 62.80 | 5.4 | 92.5 | 1290.22 | 1323.89 ± 70.89 | 5.4 | 102.6 |

[a] *Mean ± S.D., n=8*

respectively. The precision and accuracy for the E2 covering the concentration of 15-1250 pg/mL ranged from 3.4 to 12.1% and 92.5 to 108.3%, respectively. For E1 the precision ranged from 3.2 to 9.7% and the accuracy ranged between 91.1 to 108.1% as shown in Table II.

## Precision and Accuracy

The lower limit of quantification of E2 and E1 in human plasma assay was 15 pg/mL. The between-batch precision at the LLOQ was 11.5% for E2 and 11.2% for E1. The between-batch accuracy for E2 and E1 was 98.8% and 101.7% respectively (Table III). The within-batch precision for E2 and E1 was 11.6% and 10.5% respectively. The accuracy was 92.5% and 92.7% for E1 and E2 respectively.

The middle and upper quantification levels of E1 and E2 ranged from 500 to 1000 pg/mL in human plasma. For the between-batch experiment, the precision ranged from 6.3 to 8.7% and the accuracy ranged from 94.7 to 101.7% (Table III). For the within-batch experiment, the precision and accuracy

**Table III : Precision and accuracy of the LC-MS/MS method for determining estradiol and estrone concentration in plasma samples**

| Quality control concentration (pg/mL) | Within-batch precision (*n=8*) Concentration found[a] (pg/mL) | Precision (%) | Accuracy (%) | Between-batch precision (*n=3*) Concentration found[a] (pg/mL) | Precision (%) | Accuracy (%) |
|---|---|---|---|---|---|---|
| **Estradiol** | | | | | | |
| 16.04 | 14.83 ± 1.72 | 11.6 | 92.5 | 15.84 ± 1.82 | 11.5 | 98.8 |
| 40.12 | 39.23 ± 3.80 | 9.7 | 97.8 | 40.85 ± 3.22 | 7.9 | 101.8 |
| 501.53 | 522.88 ± 29.66 | 5.7 | 104.3 | 509.96 ± 34.12 | 6.7 | 101.7 |
| 1003.07 | 959.18 ± 34.48 | 3.6 | 95.6 | 949.68 ± 60.15 | 6.3 | 94.7 |
| **Estrone** | | | | | | |
| 16.51 | 15.31 ± 1.60 | 10.5 | 92.7 | 16.79 ± 1.88 | 11.2 | 101.7 |
| 41.28 | 41.43 ± 3.94 | 9.5 | 100.4 | 41.40 ± 3.06 | 7.4 | 100.3 |
| 516.09 | 533.04 ± 38.31 | 7.2 | 103.3 | 517.85 ± 43.75 | 8.4 | 100.3 |
| 1032.18 | 992.55 ± 104.94 | 10.6 | 96.2 | 1045.86 ± 91.26 | 8.7 | 101.3 |

[a] *Mean ± S.D., n=8*

for the analyte met the acceptance criteria (<±15%) and precision was below 10.6% at all concentrations tested.

The extraction recovery of E1 and E2 was 56.1 ± 1.81% on average, and the recovery ranged from 57 ± 1.81% to 59 ± 1.81%. With the moderate extraction recovery of E1 and E2, the assay has proved to be robust in high throughput analysis.

### Stability Studies

The stability of the stock solutions were determined by comparing the mean of the area responses obtained from 4 replicate analysis of aqueous standard (1567.29 pg/mL for E2 & 1612.78 pg/mL for E1) at 0.0 h and after 9.0 h. Ratio of means of area was 98.5% for drug and 98.5% for metabolite respectively. Similarly the stability of the stock solution of amlodipine was also determined and was found to be 100.8 %, which is within the acceptance range of 90 – 110%.

The Bench top stability (at room temperature) of low and high quality control samples were determined by comparing the mean of back-calculated concentrations of E2 & E1 from the freshly thawed quality control samples with those that were kept on bench top for about 9.0 hours. The ratios of means of the concentrations for the low and high QCs were 96.4% & 103.2% for E2 and 103.2% & 105.7% for E1 respectively as shown in Table IV. This was within the acceptance range of 90 – 110%.

The freeze-thaw stability was determined by measuring the assay precision and accuracy for the samples, which underwent three freeze-thaw cycles. The stability data were used to support repeat analysis. In each freeze-thaw cycle, the frozen plasma samples were thawed at room temperature for 2-3 h and refrozen for 12-24 h. After completion of each cycle the samples were analyzed and results were compared with that of zero cycle. The results showed that the analyte was stable in human plasma through three freeze-thaw cycles (Table IV). The ratios of means of the concentrations for the low and high QCs was 90.9% and 102.4% for E2 and 104% and 108.0% for E1 respectively. This was within the acceptance range of 90 – 110%. The results

**Table IV : Stability of human plasma samples of estradiol and estrone**

| Estradiol | | | | Estrone | | | |
|---|---|---|---|---|---|---|---|
| Sample concentration (pg/mL) | Concentration found[a] (pg/mL) | Precision (%) | Accuracy (%) | Sample concentration (pg/mL) | Concentration found[a] (pg/mL) | Precision (%) | Accuracy (%) |
| **Bench top stability (9 h)** | | | | | | | |
| 41.90 | 40.41 ± 0.65 | 1.6 | 96.4 | 41.69 | 41.98 ± 1.91 | 4.6 | 103.2 |
| 904.66 | 933.65 ± 30.25 | 3.2 | 103.2 | 1068.69 | 1130.08 ± 14.32 | 1.3 | 105.7 |
| **Freeze thaw stability (after 3 cycles)** | | | | | | | |
| 41.90 | 38.08 ± 2.16 | 5.7 | 90.9 | 40.69 | 43.95 ± 1.52 | 3.5 | 108.0 |
| 904.66 | 926.07 ± 12.28 | 1.3 | 102.4 | 1068.69 | 1111.28 ± 39.78 | 3.6 | 104.0 |
| **Autosampler stability (24 h)** | | | | | | | |
| 41.64 | 39.05 ± 1.06 | 2.7 | 93.8 | 42.10 | 39.11 ± 0.70 | 1.8 | 92.9 |
| 969.49 | 936.23 ± 73.69 | 7.9 | 96.6 | 1094.12 | 1074.94 ± 64.30 | 6.0 | 98.2 |

[a] *Mean ± S.D., n=6*

demonstrated that human plasma samples could be thawed and refrozen without compromising the integrity of the samples.

Stability of low and high quality control samples of E2 and E1, after processing and its internal standard in the autosampler provide advantage to determine a large number of plasma samples. Eight sets of quality control samples (low and high) were prepared and placed into the autosampler at $10^0$. They are analysed at once and three sets 25 h later. The ratio of means of the concentrations for the low and high QCs were 93.8% and 96.6% for E2 and 92.9% & 98.2% for E1 respectively as shown in Table IV. This was within the acceptance range of 90 – 110%.

The validated method has been successfully used to quantitate the estradiol and estrone in the human plasma samples for clinical patho-physiological assessment.

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