A Sensitive method for Simultaneous quantitation of Estradiol and Estrone in human plasma by Liquid chromatography-tandem mass spectrometry with electrospray ionization

Raghunatha P[1], Shital P[2], Jagruti P[2]

1: Synchron Research Services Pvt. Ltd., 6th Floor, The Chambers, SG Highway, Bodakdev, Ahmedabad – 380054
2: Department of Pharmacology, Nirma University, Sarkhej-Gandhinagar Highway, Ahmedabad - 382 481

Summary

A rapid and sensitive, sensitive liquid chromatography-tandem mass spectrometry method was developed and validated for simultaneous quantitation of Estradiol and Estrone in human plasma. The analyte were extracted by liquid-liquid extraction with dicholormethane. Amlodipine was used as the internal standard. The chromatographic separation was performed with a mobile phase comprising formic acid and methanol in gradient mode. The mass transitions of *m/z* 506.13/170.66 for 3-dansyl-estradiol, *m/z* 504.10/170.66 for 3-dansyl estrone were monitored using multipe reaction-monitoring (MRM) modes for quantification. The assay was linear over the concentration range of 10-1080 pg mL^{-1} for Estradiol and 10-1080 pg mL^{-1} for estrone. The lower level of quantification in human plasma was obtained at 10.0 pg mL^{-1} for Estradiol and 10.0 pg mL^{-1} for estrone using optimum tuning parameters. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. The validated method has been applied to analyze the Estradiol and estrone concentrations in human bioequivalence study.

Key Words: LC-MS/MS, Estradiol, Estrone, Amlodipine, Dansylation

Correspondence Address;
Raghunatha Pokale,
Synchron Research Services Pvt. Ltd.,
6th Floor, The Chambers,
SG Highway, Bodakdev,
Ahmedabad - 380054
email: raghu_nath77@yahoo.com

1. Introduction

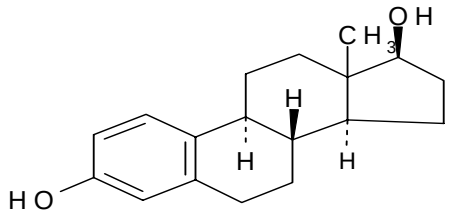
Estrogens are among the most important hormones in the female body, which pass through distant organs, interact with multiple organ systems, and play a pivotal role in the physiologic events that occur during a woman's life [1]. Estrogens are involved in development and maintenance of the female phenotype, germ cell maturation, and pregnancy. They are also important for many other, non-gender specific processes, including growth, nervous system maturation, bone metabolism/remodeling, and endothelial responsiveness.[2, 3]. It has been known that estrogens play an important role in the maintenance of bone mineral density cognitive and cardiovascular functions. They are also known to impact in developing breast, uterine, or colorectal cancer. [4,5,6,7]. Sex steroid hormones, particularly estrogens, play an important role in the pathogenesis of breast cancer. [8] In reality, after menopause, the reduced risk is gradually lost in women, suggesting that the loss of endogenous estrogens may contribute to the rapid raise of cardiovascular risk in elderly women. [9] Estrogen therapy has been available to postmenopausal women for more than 60 years. Proven benefits include relief of vasomotor symptoms and vaginal atrophy and prevention and treatment of osteoporosis. [10] The two major metabolically active estrogens in nonpregnant humans are estrone (E1), estradiol (E2) are synthesized from androgenic precursor (Δ^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[11, 12]. Presence of endogenous hormones in the plasma poses challenges for the pharmacokinetic and bioanalytical considerations in terms of endogenous baseline levels, low plasma concentration, analytical range, and possible effect of circulating hormones. [13, 14]. E1 and E2 assays based on gas-chromatography-mass spectrometry (GC-MS) address many of the shortcomings of automated imuunoassays and RIAs, but the run times may be longer than 30 min/sample, limiting throughput [15]. 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.

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. It was essential to establish an assay capable of quantifying estrogens at concentrations down to 15 pg/ml. At the same time, it was expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of estrogens. This method is simple, rapid, robust, sensitive and novel that makes it an attractive procedure in high-throughput bioanalysis for the simultaneous measurement of E1 and E2 [15].

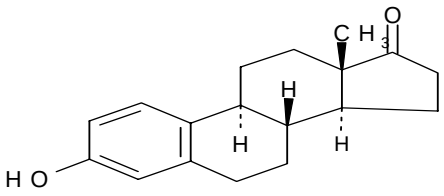
2. Experimental

2.1. Chemicals

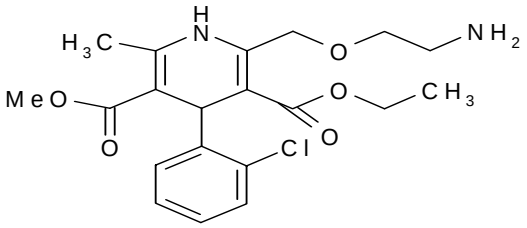
The pure substances of Estradiol, Estrone hemidhydrate were procured from the council of Europe, European Pharmacopoeia (Strasbourg, Cedex). Amlodipine besilyate procured from Sigma-Aldrich. Chemical structures are presented in Fig 1. Dansyl chloride was procured from Acros organics (New Jersey, USA). Bovine serum albumin was used as control (Sigma-Aldrich). HPLC grade methanol used for the chromatography (Sigma-Aldrich). Analytical grade sodium hydroxide, sodium hydrogen carbonate was used (Merck, India). Water was deionized using a Milli-Q system from Millipore (Bedford, MA, USA).



Estradiol



Estrone



Amlodipine

Fig.1. Chemical structures of Estradiol, Estrone and internal standard (Amlodipine)

2.2. LC-MS/MS apparatus and 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 Genesis C18 (5-µm, 50 mm X 4.6 mm i.d.) at 40 ˚C. The mobile phase composition was a mixture of 0.1 % formic acid buffer and methanol in gradient mode, with a flow rate of 0.30 mL/min.

Mass spectrometric detection was performed on ESI triple quadrupole instrument Quattro Premier (Micromass MS technologies, Waters, USA) using multiple reaction monitoring (MRM). Data processing was performed on Quan Lynx 4.0 software package (Waters).

2.3. Sample processing

A 500-µL volume of standards, control and plasma sample was transferred 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 ˚C under a stream of nitrogen. Then the residue was dissolved in 200-µL of $NaHCO_3$ (100 mM; pH 10.5). Add 200-µL dansyl chloride solution (1mg/mL in acetone), vortexed and heated for 5 min at 60 ˚C. Added 15 µL of internal standard (2.0 µg/ml), vortexed and 25- µL aliquot was injected into chromatographic system.

2.4. Bioanalytical method validation

2.4.1. Calibration and control samples

Working solutions for calibration and quality control samples were prepared from the stock solution by an adequate dilution using water/methanol (1/1 *v/v*). The IS working solution (2.0 µg/ml) was prepared by diluting its stock solution with water/methanol (1/1 *v/v*). Working solutions were added to drug free plasma to obtain the concentration levels of E2 at 10, 20, 40, 120, 420, 540, 960, 1080 pg/mL. For E1 it was at 10, 20, 40, 120, 420, 540, 960, 1080 pg/mL.

Quality control samples were prepared as a bulk, at concentration of 10.00 (lower limit of quantitation, LLOQ), 60 pg/mL (low), 540 pg/mL (medium) and 980 pg/mL (high) for E2 and concentrations of the quality control samples for E1 were 10pg/mL (lower limit of quantification, LLOQ), 60pg/mL (low), 540 pg/mL (medium) and 980 (high).

2.4.2. Calibration curve

A calibration curve of E2 and E1 were constructed from a bovine human plasma serum albumin processed without an IS, a zero sample processed with IS and eight non-zero samples covering the total range (10-1080 pg/mL), including lower limit of quantification. Eight samples of each concentration were measured. Linearity was assessed by a weighted ($1/x^2$) least squares regression analysis. The calibration curve had to have a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%.

2.4.3. Precision and accuracy

The within-batch precision and accuracy was determined by analyzing eight sets of quality control samples in a batch. The between-batch precision and accuracy was determined by analyzing eight sets of seeded quality control samples on three different batches. The quality control samples were randomized daily, processed and analyzed in position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria of within-and between-batch precision were 20% or better for LLOQ and 15% or better for the rest of concentrations and the accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for the rest of concentrations.

2.4.4. Recovery

Recovery of E1 and E2 was evaluated by comparing the mean peak areas of six extracted low, medium and high quality control samples to mean peak areas of six reference solution spiked in bovine serum albumin.

2.4.5. Stability studies

The Bench top stability (at room temperature) of low and high quality control samples were determined by comparing the mean of back-calculated concentrations from the freshly thawed quality control samples with those that were kept on bench top for about 5.0 hours.

The freeze thaw stability of low and high quality control samples were tested with three freezing periods, where the first storage of 12 hr at below -20 °C was followed by two additional periods of 12 to 24 Hrs. The percent degradation was determined by comparing the mean of back-calculated concentrations from the three freeze thaw cycles with that of a freshly thawed quality control samples.

Autosampler stability was assessed by storing the low and high quality control samples in auto sampler (5°C) for 12 hours followed by re-injecting the same samples and comparing the ratio of the mean concentrations.

3. Results 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. $[M+H]^+$ was predominant ion in the parent spectrum and was used as the precursor ion to obtain product ion spectra. The most sensitive mass transition was from *m/z* 506.13 to 170.66 for E2 and *m/z* 504.10 to 170.66 for E1 and *m/z* 509.12 to 238.23 for the IS. Thus the MRM technique was chosen for the assay development.

3.1. 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, particularly with regard to E2, and therefore pursued derivatization to improve ionization efficiency and experimental sensitivity. Derivatization with dansyl chloride was highly effective and to cope with the matrix effect due to endogenous drug substances, bovine serum albumin was used as control in the validation studies and the Lower level of the calibration range was set slightly higher than the base line value.

Different mobile phases consisting of water-acetonitrile or water-methanol 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 methanol 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 coelution of E2, E1 and IS, paying attention to matrix effects as well as good peak shapes. A high proportion of organic solvent (0.1% formic acid/methanol 15/85, v/v) was used to coelute E2 and E1 at retention time of 5.22 and 5.01 min respectively. Flow rate of 3.0 mL/min produced a good peak shape and brought the runtime to 6 min. The method was subjected to specificity and matrix effect to establish the ability of mass spectrometer to separate and quantify the analyte in the presence of endogenous species and co-administered medications.

3.2. Internal standard(IS)

The chromatographic conditions and ionization properties of amlodipine (*m/z* 509.12/238.23) was found to match with that of the estrogens and hence was used as internal standard (external method)

3.3. Calibration curves

Calibration curve was linear over the concentration range of 10-1080pg/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 0.996 and 0.990 for E2 and E1 respectively (Table 1 & 2). The precision and accuracy for the E2 covering the concentration of 10-1080 pg/mL ranged from 2.4 to 10.1% and 94.13 to 113.12%, respectively. For E1 the precision ranged from 2.9 to 9.4% and the accuracy ranged between 46.9 to 105.3%.

The calibration curve obtained as described above was suitable for generation of acceptable data for the concentration of the E2 and E1 in the samples during the validation.

Concentrations added (pg/mL)	Concentration found(mean±SEM, *n*=8) pg/mL	Precision (%)	Accuracy (%)
10	11.00±0.94	5.0	100.3
20	21.00±2.79	10.1	98.24
40	41.00±4.20	9.0	94.13
120	122±9.40	3.8	113.12
420	388.95±23.85	2.5	104.44
540	543.58±33.05	5.5	67.73
960	950.67±23.81	2.4	101.13
1080	1090.13±42.80	6.4	98.52

Table 1: Precision and accuracy data of back-calculated concentrations of calibration samples for Estradiol (E2) in human plasma

Concentrations added (pg/mL)	Concentration found (mean±SEM, *n*=8) pg/mL	Precision (%)	Accuracy (%)
10	10.72±0.59	5.2	100.6
20	20.87±1.98	6.7	85.6
40	40.62±2.32	9.4	89.1
120	120.83±8.81	5.2	93.1
420	418±2.81	3.6	103.4
540	545±15.05	8.4	46.9
960	957.04±30.31	2.9	105.3
1080	1112.89±70.89	5.4	103.4

Table 2: Precision and accuracy data of back-calculated concentrations of calibration samples for Estrone (E1) in human plasma

3.4. Specificity

LC-MS/MS analysis of the blank human plasma samples showed the presence of endogenous E2 and E1 and no interference with the IS. Hence the specificity of the method was established by comparing with the spiked

bovine serum albumin (control). Representative chromatograms of extracted blank plasma (Fig. 2), demonstrating the specificity and selectivity of the method. No significant interference in the blank plasma traces was seen from endogenous substances in drug-free human plasma at the retention time of the analytes and internal standard. Fig 3 depicts a representative ion-chromatogram for the LLOQ (10 pg/mL) of the calibration curve. Excellent sensitivity was observed for 25- µL injection volume.

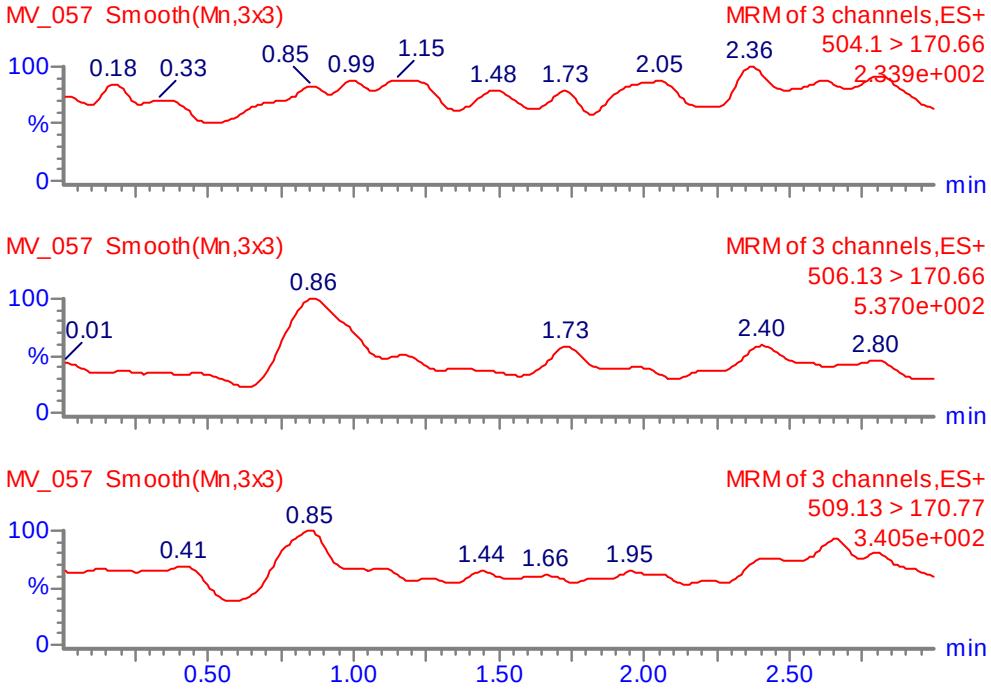


Fig.2 Chromatograms of blank human plasma (drug and internal standard free) for E1, E2 & IS

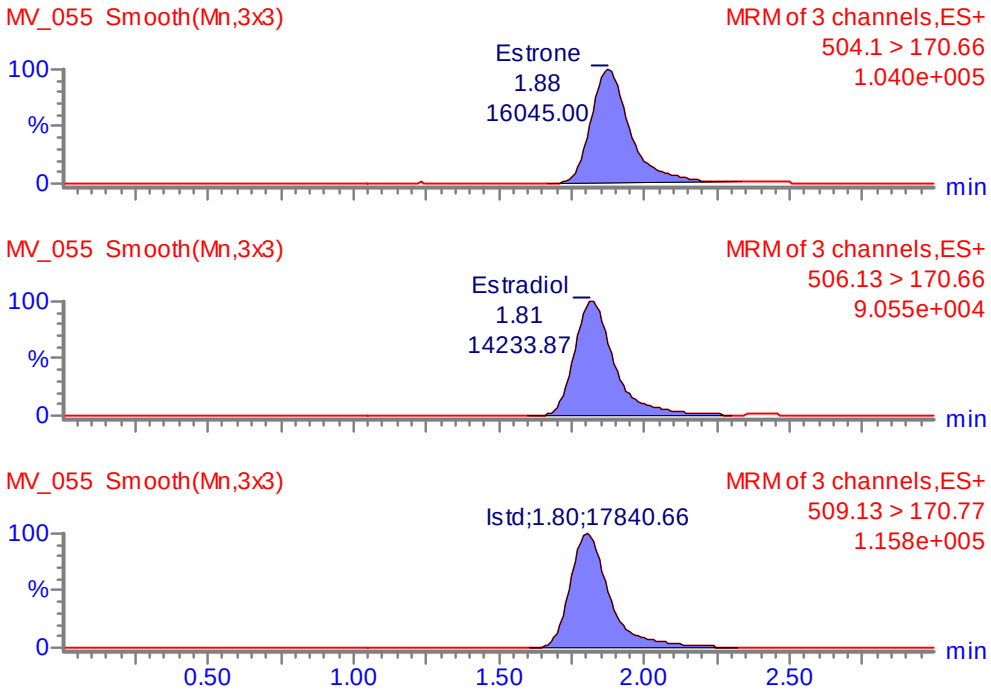


Fig.3. Representative Chromatogram of LLOQ in plasma containing 10 pg/mL of E1, E2 & IS

3.5. Matrix effect

The matrix effects in the LC-MS/MS method were evaluated by spiking bovine albumin serum samples with low and high QC samples. Six independent plasma lots were used with six samples from each lot. Percent nominal concentrations estimated were well within the acceptable limits. Hence the effect of matrix on estimation of drug is negligible.

3.3. Extraction recovery

The extraction recovery of E1 and E2 was 64.1 ± 5.81% on average, and the recovery ranges from 63.01 ± 2.81% to 6.105± 3.84%. With the moderate extraction recovery of E1 and E2, the assay has proved to be robust in high throughput analysis.

3.7. Lowest concentration

The lower limit of quantification of E2 and E1 in human plasma assay was 10 pg/mL. The between-batch precision at the LLOQ was 8.7% for E2 and 11.7% for E1. The between-batch accuracy for E2 and E1 was 98.7% and 100.3% respectively (Table 3 &4). The within-batch precision for E2 and E1 was 4.7% and 11.6% respectively. The accuracy was 98.8% and 90.2% for E1 and E2 respectively.

Concentration added (pg/mL)	Within-batch precision (*n=4*)			Between-batch precision (*n*=4)		
	Concentration found (mean**±SEM,** pg/mL	Precision (%)	Accuracy (%)	Concentration found (mean±SEM) pg/mL	Precision (%)	Accuracy (%)
10.00	10.83**±**2.72	3.6	93.5	10.84**±**1.82	5.5	98.23
60.00	58.34**±**6.80	4.7	98.8	62.85**±**3.22	4.9	102.8
540.00	543.45**±**12.66	2.7	102.3	546.96**±**29.12	8.7	98.7
980.00	975.54**±**24.48	1.6	98.6	969.68**±**38.15	9.3	94.7

Table 3: Precision and accuracy of the LC-MS/MS method for determining Estradiol concentration in plasma samples

Concentration added (pg/mL)	Within-batch precision (*n=4*)			Between-batch precision (*n*=4)		
	Concentration found (mean**±SEM,** pg/mL	Precision (%)	Accuracy (%)	Concentration found (mean±SEM) pg/mL	Precision (%)	Accuracy (%)
10.00	10.21**±2**6.60	9.5	91.7	10.29**±**1.88	9.2	106.7
60.00	63.43**±**16.94	8.5	92.4	62.40**±**3.06	8.4	96.3
540.00	533.04**±**30.00	5.2	107.3	527.85**±**43.75	6.4	98.3
980.00	992.55**±**104.94	11.6	90.2	976.86**±**91.26	11.7	100.3

Table 4: Precision and accuracy of the LC-MS/MS method for determining estrone concentration in plasma sample

3.8. Middle and upper concentrations

The middle and upper quantification levels of E1 and E2 ranged from 540 to 980 pg/mL in human plasma. For the between-batch experiment, the precision ranged from 5.3 to 6.7% and the accuracy ranged from 96.7 to 104.7% (Table 4, 5). For the within-batch experiment, the precision and accuracy for the analyte met the acceptance criteria (<±15%) and precision was below 8.6% at all concentrations tested.

3.9. Stability

The stability of the stock solutions were determined by comparing the mean of the area responses obtained from 6 replicate analysis of aqueous standard (540 pg/mL for E2 & E1) after 7.0 hours. Ratio of means of area was 97.15% for drug and 99.15% for metabolite respectively which is within the acceptance range of 90 – 110%.

The stability of the stock solution of amlodipine was determined by comparing the mean of the area responses obtained from 6 replicate analysis of aqueous standard after 9.0 hours. Ratio of means of area was 100.9 %, which is within the acceptance range of 90 – 110%.

The freeze-thaw stability was determined by measuring the assay precision and accuracy for the samples (Table 5 & 6), 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 1.0 h and refrozen for 12-24 hr. 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 5 & 6). The ratios of means of the concentrations for the low and high QCs was 90.9% and 105.4% for E2 and 94% and 109% for E1 respectively. This was within the acceptance range of 90 – 110%. The results demonstrated that human plasma samples could be thawed and refrozen without compromising the integrity of the samples.

Sample concentration (pg/mL) (*n*=6)	Concentration found (Mean**±SEM**) (pg/mL)	Precision (%)	Accuracy (%)
Bench top stability (11h)			
60.00	60.41**±**0.65	2.6	95.47
980.00	963.65**±**40.25	5.7	105.2
Freeze thaw stability (after 3 cycles)			
60.00	68.08**±**6.16	5.7	90.9
980.00	966.07**±**12.28	6.3	105.4
Autosampler stability (28 h)			
60.00	59.05**±**2.06	3.7	96.8
98.00	986.23**±**23.69	7.9	98.6

Table 5: Stability of human plasma samples of Estradiol (E2)

Sample concentration (pg/mL) (*n*=6)	Concentration found (mean**±SEM**) (pg/mL)	Precision (%)	Accuracy (%)
Bench top stability (11 h)			
60.00	61.98**±**5.91	7.6	106.2
980.00	970.08**±**4.32	3.3	102.7
Freeze thaw stability (after 3 cycles)			
60.00	60.95**±**3.52	5.5	109.0
980	980.28**±**49.78	4.6	94.0
Autosampler stability (28 h)			
60.00	62.11**±**3.70	3.8	95.9
980.00	989.94**±**14.30	4.0	96.2

Table 6: Stability of human plasma samples of Estrone (E1)

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. two sets of quality control samples (low and high) were prepared as described in Section 2.3, and placed into the autosampler to 5 ˚C. They are analysed after 12 hr later. The ratio of means of the concentrations for the low and high QCs were

96.8% and 98.6% for E2 and 95.9% & 96.2% for E1 respectively. This was within the acceptance range of 90 – 110%.

Differences in responses of spiked standards at time 0 h and after 28 h for E1 and E2 were less than 5%. The significance was defined if the percent change in response between 0 and 24 h is more than 9%. Therefore, the differences in responses were not significant, indicating the stability of E2 and E1 at room temperature upto 25 h. Further, the analyte was found to be stable after reconstitution in diluent for at least 12 h at 4 ˚C. The re-injection reproducibility was established to determine if an analytical batch could be reanalyzed in case of an unexpected delay in analyses. The same set of QC samples were repeated after the analysis with a 3 h gap between, during which the samples were stored at 4 ˚C, and in all cases the deviations were less than 15%. On similar lines, stability of the extracted dry residues was also established to be over 24 h (deviations observed <10%). In addition, the stock solutions of E1 and E2 and IS were also found to be stable for at least 3 months at 4 ˚C.

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 11.0 hours. The ratios of means of the concentrations for the low and high QCs were 95.47% & 105.2% for E2 and 102.7% & 100.02% for E1 respectively. This was within the acceptance range of 90 – 110%.

4. Conclusions

In summary, the method described here for the quantification of estrone and estradiol from human plasma by LC-MS/MS in positive ionization mode using multiple reaction monitoring. The current method has shown acceptable precision and adequate sensitivity for the quantification of E1 and E2 in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. Furthermore, it was utilized for the analysis of subject samples. The method described is simple, rapid, sensitive, specific and fully validated as per FDA guidelines. The cost-effectiveness, simplicity and speed of liquid/liquid

extraction and simultaneous quantification of estrone and estadiol make it an attractive procedure in the bioanalysis of estrogens. The validated method allows the quantitation of estrogen in the 10-1080 pg/mL range.

5. Acknowledgement

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