

# Determination of 17 $\beta$ -Estradiol in Rabbit Plasma by Gas Chromatography with Flame Ionization Detection

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Yilmaz and Kadioglu: Determination of 17  $\beta$ -Estradiol in Rabbit Plasma

This article describes gas chromatography-flame ionization detection method for determination of 17  $\beta$ -estradiol in rabbit plasma. 17  $\beta$ -estradiol and internal standard progesterone were extracted from plasma using liquid-liquid extraction method. Linearity was found between 0.25 and 20  $\mu\text{g}/\text{ml}$  ( $r^2=0.994$ ) for plasma samples. Intra-day and inter-day precision, expressed as the relative standard deviation were less than 5.5%, and accuracy (relative error) was less than 3.5%. The mean recovery of 17  $\beta$ -estradiol samples was 94.4%. The limits of detection and quantification of method for plasma samples were 0.10  $\mu\text{g}/\text{ml}$  and 0.15  $\mu\text{g}/\text{ml}$ , respectively. Also, clinically used other 10 drugs were investigated to check for potential interferences and the method was successfully applied to the determination of 17  $\beta$ -estradiol in New Zealand white rabbits.

**Key words:** Flame ionization detection, gas chromatography, liquid-liquid extraction, pharmacokinetics, validation, 17  $\beta$ -estradiol

17  $\beta$ -estradiol (fig. 1) is the most potent of the natural human estrogens<sup>[1]</sup>. It is the most potent estrogen of a group of endogenous estrogen steroids which includes estrone and estriol. It is responsible for the growth of breast and reproductive epithelia, maturation of long bones, and development of secondary sexual characteristics. 17  $\beta$ -estradiol and its semi-synthetic esters are primarily used as menopausal hormones. It may also be used as replacement therapy for female hypogonadism or primary ovarian failure. The decrease of 17  $\beta$ -estradiol at menopause is often accompanied by vascular instability and an increasing risk of osteoporosis<sup>[2]</sup>.

Several methods have been reported for the determination of 17  $\beta$ -estradiol including voltammetry<sup>[3]</sup>, high performance liquid chromatography (HPLC)<sup>[1,4-8]</sup>, liquid chromatography-tandem mass spectrometry (LC-MS-MS)<sup>[9]</sup> and gas chromatography-mass spectrophotometry (GC-MS)<sup>[10-15]</sup>.

In addition, no method is reported till date for determination of 17  $\beta$ -estradiol by gas chromatography-flame ionization detection (GC-FID) in rabbit plasma. Therefore, the present work

describes the determination of 17  $\beta$ -estradiol in rabbit plasma. The developed method was validated by sensitivity, linearity, precision, accuracy and stability parameters according to the Center for Drug Evaluation and Research (CDER) guidance for bio-analytical method validation<sup>[16]</sup>.

The advantages of the present method include simple and single step extraction procedure using inexpensive chemicals and short run time. Also, this method was used to assay the 17  $\beta$ -estradiol in plasma samples obtained from three rabbits which had been given as an oral tablet of Estrofem tablet (4 mg/kg, 17  $\beta$ -estradiol).

## MATERIALS AND METHODS

17  $\beta$ -estradiol and progesterone (internal standard [IS]) were purchased from Sigma (St. Louis, Mo, USA).

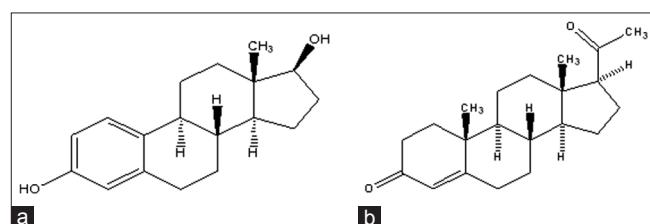


Fig. 1: Chemical structures of analytes.

Chemical structures of (a) 17  $\beta$ -estradiol and (b) progesterone (IS)

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Ethylacetate, hexane, methanol, ethanol and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of analytical grade. Distilled water was prepared as required using aquaMAX™ ultra, Young instrument (Korea) ultrawater purification system. Estrofem tablet containing 17  $\beta$ -estradiol was obtained from the local market (Erzurum, Turkey). All gases were supplied by Havas (Erzurum, Turkey).

Chromatographic analyses were carried out on a Agilent 6890N Network gas chromatography system equipped with a flame ionization detector. HP-5 column with 0.25  $\mu\text{m}$  film thickness (30 m $\times$ 0.320 mm I.D., USA) was used for separation.

Injection and detector temperature is 250 and 300°, respectively. Splitless injection was used. The carrier gas ( $\text{N}_2$ ) flow-rate was kept constant during the run at 2 ml/min. Nitrogen (30 ml/min), hydrogen (44 ml/min) and dry air (400 ml/min) were used as auxiliary gases for the flame ionization detector.

#### **Preparation of the standard and quality control solutions:**

Calibration standards of 17  $\beta$ -estradiol at concentrations of 0.25, 0.50, 0.75, 1.25, 2.5, 5, 7.5, 10, 12.5, 15 and 20  $\mu\text{g}/\text{ml}$  were prepared by spiking appropriate amount of the standard solutions in blank plasma. Standard solutions were stored at -20°. IS stock solution was made at an initial concentration of 100  $\mu\text{g}/\text{ml}$ . The IS working solution (30  $\mu\text{g}/\text{ml}$ ) was made from the stock solution using methanol dilution.

The concentrations of 17  $\beta$ -estradiol were 1, 9 and 17.5  $\mu\text{g}/\text{ml}$  rabbit plasma to represent low, middle and high quality controls, respectively. Appropriate volumes from stock solution of 17  $\beta$ -estradiol were added to normal rabbit plasma to get low, middle and high quality control samples, respectively, and stored at -20°. The quality control samples were taken out from storage for analysis to determine intra- and inter-day precision and accuracy.

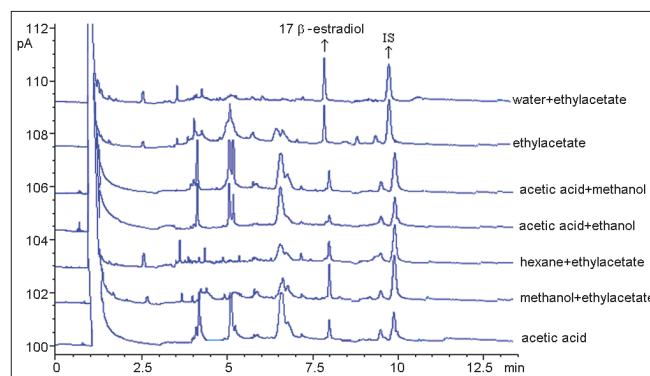
#### **Plasma sample preparation procedure:**

Various solvents were tested for the extraction of 17  $\beta$ -estradiol in our laboratory. They were water+ethylacetate, ethylacetate, acetic acid+methanol, acetic acid+ethanol, hexane+ethylacetate, methanol+ethylacetate and acetic acid (fig. 2). Water and ethylacetate solvent mixture was selected for liquid-liquid extraction method as shown in Table 1.

Ethylacetate is an extensively used extracting solvent because of its high polarity and volatility<sup>[14]</sup>. The solvent ethylacetate gave good recovery and the absolute recoveries of 17  $\beta$ -estradiol and IS from rabbit plasma. No interfering peak and ghost peak were detected in the blank rabbit plasma in analysis (fig. 3c).

The use of IS is very important. When, IS is not available commercially, an alternative approach has been used. IS chosen should match the chromatographic properties, recovery and chemical properties of the analyte. Progesteron was found to match these criteria, and therefore was chosen as an IS. Other IS's such as growth hormone, estriol, diazepam, atenolol, carbamazepine,  $\alpha$ -tocopherol, medazepam and prilocaine were also tried, but were rejected because of their low recovery and inefficient extraction. Progesteron was selected because of its high recovery as IS. Good chromatographic separation was obtained.

Blood samples were collected into the tubes containing disodium ethylenediaminetetraacetic acid (EDTA) and centrifuged at 4500 $\times g$  for 10 min. A 0.2 ml of the resultant plasma samples was spiked with 0.1 ml of 17  $\beta$ -estradiol and 0.1 ml of IS. After vortex mixing for 5



**Fig. 2: Overlain chromatograms of various solvents tried for extraction.**

Chromatogram of 17  $\beta$ -estradiol (5  $\mu\text{g}/\text{ml}$ ) and IS (3  $\mu\text{g}/\text{ml}$ ) by liquid-liquid extraction method using different solvents in rabbit plasma

**TABLE 1: RECOVERIES OF 17  $\beta$ -ESTRADIOL BY LIQUID-LIQUID EXTRACTION METHOD USING DIFFERENT SOLVENTS IN RABBIT PLASMA**

Solvent	% Recovery
Water+ethylacetate	97.4
Ethylacetate	92.8
Acetic acid+methanol	72.4
Acetic acid+ethanol	84.5
Hexane+ethylacetate	84.3
Methanol+ethylacetate	86.9
Acetic acid	69.8

s, 1.9 ml water and then 5 ml ethyl acetate was added. The mixture was vortexed for 10 s and then centrifuged at 3000×g for 3 min. The organic phase was transferred into another tube and evaporated to dryness at room temperature under nitrogen gas. The dry residue was dissolved in 1 ml methanol. Two microlitre volumes were injected into the GC-FID system.

#### Method development:

The method development for the assay of 17 β-estradiol was based on its chemical properties. The column and acquisition parameters were chosen to be a starting point for the method development. 17 β-estradiol is a polar molecule. Therefore, the capillary column coated with 5% phenyl and 95% dimethylpolysiloxane is an adequate choice for separation of 17 β-estradiol.

The GC-FID parameters used in the method development were based on the boiling point. The injection port and detector temperature was set to 250 and 300°, respectively<sup>[14]</sup>. Different temperature programs were investigated for method. At the end of this investigation, the temperature program of the GC-FID was as follows: Initial temperature was 150°, held for 1.5 min, increased to 260° at a rate of 50°/min held for 5 min, and finally to 270°/min at a rate of 10°/min and held for 3.3 min. The head pressure was set to ensure a hydrogen flow of 44 ml/min. The splitless mode was chosen. The solvent, column and acquisition parameters were chosen to be a starting point for the method development. In addition, preliminary precision and linearity studies performed during the development of the method showed that the 2 µl injection volume was reproducible and the peak response was significant at the analytical concentration chosen. Typical 17 β-estradiol and IS chromatogram obtained in rabbit plasma is presented in fig. 3.

#### Specificity:

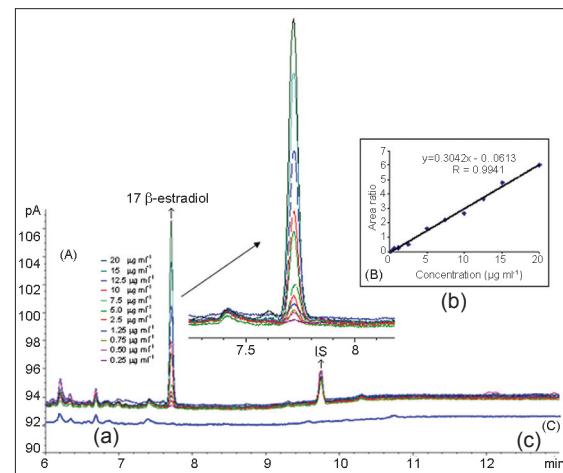
It was determined that liquid-phase extraction process was necessary at the sample preparation procedure. Several solvents (ethylacetate, hexane, dichloromethane, acetonitrile, butanol and chloroform) were tested for the extraction. Finally, ethylacetate and water mixture proved to be the most efficient in extracting 17 β-estradiol from rabbit plasma. There was no interference in the chromatogram of blank rabbit plasma. The retention times of 17 β-estradiol and IS in plasma were approximately 7.8 and 9.8 min, respectively (fig. 3a, c).

#### Linearity:

Eleven 17 β-estradiol standard samples at different concentration levels ranging from 0.25 to 20 µg/ml with constant concentration of IS (3 µg/ml) in spiked rabbit plasma samples were prepared and injected onto the GC-FID system. The calibration curve (fig. 3b) was established by plotting the ratio of the peak areas of 17 β-estradiol and IS obtained after extraction of the spiked rabbit plasma sample. The regression equations were calculated from the calibration graphs, along with the standard deviations of the slope and intercept on the ordinate [Table 2].

#### Precision and accuracy:

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by analysing spiked blank rabbit plasma six times per day, at three different concentrations (1, 9, 17.5 µg/ml). The intermediate precision was evaluated by analysing the same plasma samples once daily for 3 days. The relative standard deviation (RSD) of the predicted concentrations from the regression equation was



**Fig. 3: Linearity of the method**  
**(a)** Chromatogram of obtained calibration graph point using the assay spiked procedure, **(b)** Mean calibration graph ( $n=6$ ), **(c)** Blank rabbit plasma

**TABLE 2: LINEARITY OF 17 β-ESTRADIOL IN RABBIT PLASMA**

Linearity (µg/ml)	0.25-20
Regression equation <sup>a</sup>	$y=0.3042x-0.0613$
Standard deviation of slope	$8.99 \times 10^{-3}$
Standard deviation of intercept	$3.14 \times 10^{-2}$
Correlation coefficient	0.9941
Standard deviation of correlation coefficient	$1.50 \times 10^{-3}$
Limit of detection (µg/ml)	0.10
Limit of quantification (µg/ml)	0.15

<sup>a</sup>Based on six calibration curves, y: peak-area ratio, x: 17 b-estradiol concentration (µg/ml)

**TABLE 3: PRECISION AND ACCURACY OF 17 β-ESTRADIOL IN RABBIT PLASMA (N=6)**

Added (μg/ml)	Intra-day			Inter-day		
	Found (Mean±SD <sup>a</sup> )	Precision% RSD <sup>b</sup>	Accuracy <sup>c</sup>	Found (Mean±SD <sup>a</sup> )	Precision % RSD <sup>b</sup>	Accuracy <sup>c</sup>
1	0.98±0.046	4.69	-2.00	0.97±0.050	5.15	-3.00
9	9.20±0.285	3.09	2.22	9.30±0.446	4.79	3.33
17.5	16.90±0.439	2.59	-3.43	17.10±0.633	3.70	-2.34

SD<sup>a</sup> = Standard deviation of six replicate determinations; RSD = Relative standard deviation; <sup>b</sup>Average of six replicate determinations; Accuracy<sup>c</sup> = (% relative error) (found-added)/added ×100

taken as precision. The accuracy of this method was assessed as the percentage relative error (% relative error=[(found-added concentration)/added concentration]×100). For all the concentrations studied, intra- and inter-day RSD values were ≤5.15% and for all concentrations of 17 β-estradiol the relative errors were ≤3.43%. These results are given in Table 3.

#### Limits of detection and quantification:

The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise, but not quantitated. The limit of quantification (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOD and LOQ were studied to test the sensitivity of the method. The LOD was defined as a signal/noise ratio of 3:1 and the LOQ was with a S/N ratio of 10<sup>[17]</sup>. The LOD and LOQ values for 17 β-estradiol were found to be 0.10 and 0.15 μg/ml, respectively (Table 2).

#### Recovery:

The analytical recovery of 17 β-estradiol from rabbit plasma was assessed by direct comparison of concentrations of 17 β-estradiol obtained after the whole extraction procedure using six replicate at 11 concentrations levels (0.25, 0.50, 0.75, 1.25, 2.5, 5, 10, 12.5, 15 and 20 μg/ml) in the calibration graph versus standard 17 β-estradiol solutions. The extraction recoveries of 17 β-estradiol from rabbit plasma were between 88.0 and 101.6% as shown in Table 4.

#### Stability:

For the determination of the stability of 17 β-estradiol in plasma at room temperature, 4° and -20° refrigeration temperature, low (2.5 μg/ml) and high (15 μg/ml). 17 β-estradiol concentrations were kept for 24 h and 3 days. Then the stability measurements were carried out. Rabbit plasma samples were found to be stable after 3 days with no significant change in concentration when stored at 4 and -20°. The results of these stability studies are given in Table 5, where

**TABLE 4: RECOVERY OF 17 β-ESTRADIOL IN RABBIT PLASMA (N=6)**

Added (μg/ml)	Found (Mean±SD <sup>a</sup> )	% Recovery	% RSD <sup>b</sup>
0.25	0.22±0.014	88.0	6.36
0.50	0.46±0.026	92.0	5.65
0.75	0.72±0.037	96.0	5.14
1.25	1.16±0.049	92.8	4.22
2.5	2.36±0.108	94.4	4.58
5	4.78±0.185	95.6	3.87
10	10.16±0.369	101.6	3.63
12.5	11.85±0.472	94.8	3.98

SD<sup>a</sup>: Standard deviation of six replicate determinations RSD: Relative standard deviation <sup>b</sup>Average of six replicate determinations

the percent recovery ratios are within the acceptance range of 90-110%.

#### Interference study:

In order to assess the possible analytical applications of the proposed method, the effect of common drugs used in patients was studied by analysing spiked plasma samples. Commonly prescribed drugs Grandpherol (alpha-tocopherol), Calcimax (vitamin D<sub>3</sub>), Yasmin (ethinyl estradiol), Apranax (naproxen sodium), Climen (estradiol valerate), Epdantoin (phenytoin sodium), Romaryd (rofecoxib), Farlutal (medroxy progesterone), Ephynal (alpha-tocopherol acetate), Dolorex (diclofenac) were analysed for possible interference. No interference was observed under the chromatographic conditions. These results are given in fig. 4.

#### Application of the method:

Prior to the study, the ethical protocol was approved by the Ethical Committee for Medical Experimental Research and Application Centre, Ataturk University.

Three rabbits were housed with free access to food and water, except for the final 2 h before the experiment. After a single oral administration of 4 mg/kg of Estrofem tablet, 1.0 ml of blood samples were collected from the marginal ear vein at 0, 15, 30, 60, 120, 180, 240, 360, 420 and 480 min time-points into EDTA collection tubes. The blood was immediately centrifuged 6000 rpm for 10 min at ambient temperature. The supernatant plasma

layer was separated and analysed for 17  $\beta$ -estradiol concentrations as described above.

The maximum plasma concentration ( $C_{\max}$ ) and the time to reach maximum concentration ( $T_{\max}$ ) were directly determined from the plasma concentration versus time curves. The area under the curve from 0 to  $t$  ( $AUC_{0-t}$ ) was calculated by the linear trapezoidal rule. The area under the curve from 0 h to infinity ( $AUC_{0-\infty}$ ) was estimated by summing the area from 0 to  $t$  ( $AUC_{0-t}$ ) and  $t$  to infinity ( $AUC_{t-\infty}$ ), where  $AUC_{t-\infty} = C_t/K_{el}$ , with  $C_t$  defined as the last measured plasma concentration at time  $t$ , and  $k_{el}$  the slope of the terminal portion of the ln (plasma concentration) versus time curve. The elimination half-life ( $t_{1/2}$ ) was calculated using the pharmacokinetic relationship  $t_{1/2} = \ln(2)/k_{el}$ <sup>[18]</sup>.

GC-FID chromatogram of rabbit plasma after oral administration of Estrofem tablet is shown in fig. 5. Representative mean plasma concentrations versus time profiles following oral administration of 17  $\beta$ -estradiol to three rabbits are presented in fig. 6.

## RESULTS AND DISCUSSION

Ingrand *et al.*<sup>[9]</sup> has reported LC method with tandem mass detection for the analysis of 17- $\beta$  estradiol in effluents of wastewater treatment plants. 17- $\beta$  estradiol is extracted via solid-phase extraction using C<sub>18</sub> cartridge. The calibration curve of LC-MS-MS method was linear for 17- $\beta$  estradiol in the range 10-50 ng/l. Intra- and inter-day precision values were lower than 15%. The maximum recovery of 17- $\beta$  estradiol was 84%. The LOQ of method was found 10 ng/l. Detection using LC-MS-MS would be a more sensitive approach, but is costly and not yet available for every laboratory.

GC-FID method (proposed method) is a little simpler and faster with respect to the deproteinization step and analysis time. In this study, the recovery percentage of 17- $\beta$  estradiol is higher than other reported method<sup>[9,10]</sup>. There is not a derivatization process. Also, the extraction processes do not take

much time<sup>[4,10-13]</sup>; additionally, the retention time is short which is an advantage<sup>[4,10,12,13]</sup>.

17- $\beta$  estradiol was extracted with a solid phase extraction procedure in other techniques<sup>[4,10-13]</sup>. These methods are also the most comprehensive method which can extract 17- $\beta$  estradiol in a single extraction procedure.

In conclusion, a new rapid, simple GC-FID method has been developed for the determination of 17

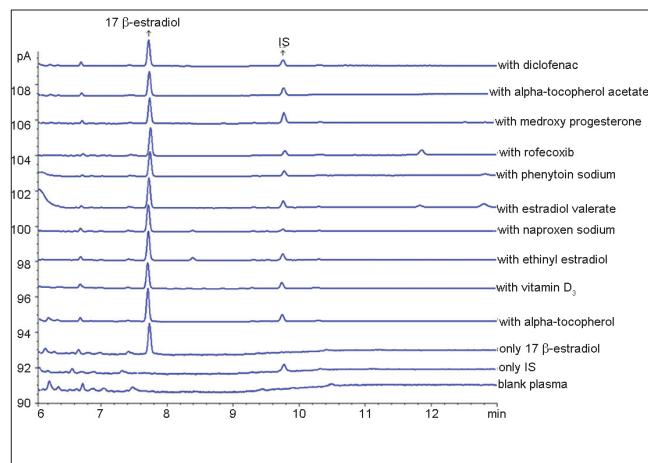


Fig. 4: Overlaid chromatograms for specificity.  
Chromatogram of obtained blank rabbit plasma, IS (3  $\mu$ g/ml), 17  $\beta$ -estradiol (20  $\mu$ g/ml) and 17  $\beta$ -estradiol with interference substances, [Grandpheral (alpha-tocopherol), Calcimax (vitamin D3), Yasmin (ethynodiol diacetate), Apranax (naproxen sodium), Climen (estradiol valerate), Epdantoin (phenytoin sodium), Romaryd (rofecoxib), Farlutal (medroxy progesterone), Ephynal (alpha-tocopherol acetate), Dolorex (diclofenac)]

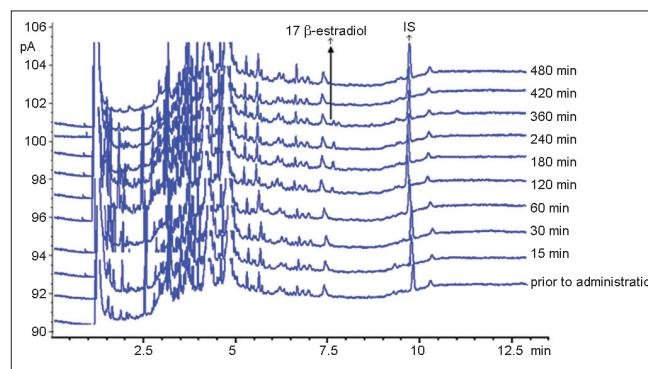
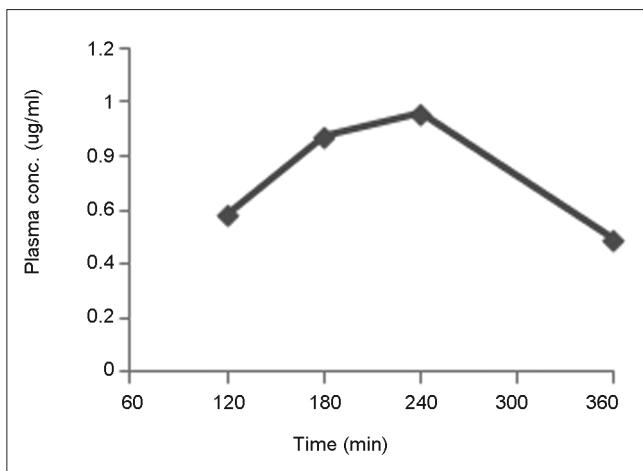


Fig. 5: Overlaid chromatograms of Pharmacokinetic samples.  
Chromatogram of samples collected from rabbit prior to and at 15, 30, 60, 120, 180, 240, 360, 420 and 480 min after oral administration of 4 mg/kg of Estrofem

TABLE 5: STABILITY OF 17  $\beta$ -ESTRADIOL IN RABBIT PLASMA

Concentration (mg/ml)	Room temperature 24 h	Room temperature 72 h	Refrigeratory 4°, 24 h	Refrigeratory 4°, 72 h	Frozen -20°, 24 h	Frozen -20°, 72 h
2.5	2.44±5.12	2.43±7.18	2.46±3.16	2.44±5.23	2.47±2.54	2.47±4.97
15	14.9±4.36	14.8±6.29	15.2±3.46	15.45±4.62	15.17±2.89	15.34±3.26



**Fig. 6: Mean plasma concentration-time profile of 17  $\beta$ -estradiol for three rabbits**

$\beta$ -estradiol in rabbit plasma. Also, the method was completely validated by sensitivity, stability, specificity, linearity, accuracy and precision parameters for determination of 17  $\beta$ -estradiol in rabbit plasma. The method was found to be linear over an analytical range of 0.25-20  $\mu\text{g}/\text{ml}$ . Additional advantages of this method include small sample volume (0.2 ml), good extraction recovery from plasma and a readily available IS. To our knowledge, this is the first description of 17  $\beta$ -estradiol pharmacokinetics in rabbit plasma by GC-FID method in the literature. It can be very useful and an alternate to performing pharmacokinetic studies in determination of 17  $\beta$ -estradiol for clinical use.

## ACKNOWLEDGEMENTS

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## REFERENCES

- Russell JA, Malcolm RK, Campbell K, Woolfson AD. High-performance liquid chromatographic determination of 17 $\beta$ -estradiol and 17 $\beta$ -estradiol-3-acetate solubilities and diffusion coefficients in silicone elastomeric intravaginal rings. *J Chromatogr B Biomed Sci Appl* 2000;744:157-63.
- Havlíková L, Nováková L, Matysová L, Sícha J, Solich P. Determination of estradiol and its degradation products by liquid chromatography. *J Chromatogr A* 2006;1119:216-23.
- Salci B, Biryol I. Voltammetric investigation of beta-estradiol. *J Pharm Biomed Anal* 2002;28:753-9.
- López de Alda MJ, Barceló D. Determination of steroid sex hormones and related synthetic compounds considered as endocrine disrupters in water by fully automated on-line solid-phase extraction-liquid chromatography-diode array detection. *J Chromatogr A* 2001;911:203-10.
- Kimura A, Taguchi M, Arai H, Hiratsuka H, Namba H, Kojima T. Radiation-induced decomposition of trace amounts of  $\beta$ -estradiol in water. *Radiat Phys Chem* 2004;69:295-301.
- Terada H, Yamamoto K, Miyabe M. Determination of corticosteroids and anabolic agents in health food by high performance liquid chromatography. *Japan J Toxicol and Environ Health* 1992;38:537-44.
- Mao L, Sun C, Zhang H, Wang X, Li Y, Wu D. Determination of 17 $\alpha$ -estradiol and 17 $\beta$ -estradiol in urine by high performance liquid chromatography with pre-column derivatization. *Fenxi Huaxue* 2003;31:1446-9.
- Nygaard L, Drøhse Kilde H, Andersen SG, Henriksen L, Overby V. Development and validation of a reversed-phase liquid chromatographic method for analysis of degradation products of estradiol in Vagifem tablets. *J Pharm Biomed Anal* 2004;34:265-76.
- Ingrand V, Henry G, Beausse J, de Roubin MR. Analysis of steroid hormones in effluents of wastewater treatment plants by liquid chromatography-tandem mass spectrometry. *J Chromatogr A* 2003;1020:99-104.
- Zacharia LC, Dubey RK, Jackson EK. A gas chromatography/mass spectrometry assay to measure estradiol, catecholestradiols, and methoxyestradiols in plasma. *Steroids* 2004;69:255-61.
- Fotsis T, Adlercreutz H. The multicomponent analysis of estrogens in urine by ion exchange chromatography and GC-MS-I. Quantitation of estrogens after initial hydrolysis of conjugates. *J Steroid Biochem* 1987;28:203-13.
- Quintana JB, Carpintero J, Rodríguez I, Lorenzo RA, Carro AM, Cela R. Determination of natural and synthetic estrogens in water by gas chromatography with mass spectrometric detection. *J Chromatogr A* 2004;1024:177-85.
- Kawaguchi M, Ito R, Sakai N, Okanouchi N, Saito K, Nakazawa H. Dual derivatization-stir bar sorptive extraction-thermal desorption-gas chromatography-mass spectrometry for determination of 17  $\beta$ -estradiol in water sample. *J Chromatogr A* 2006;1105:140-7.
- Fedeniuk RW, Boison JO, MacNeil JD. Validation of a gas chromatography-mass spectrometry method for the determination of pg/ml levels of 17beta-estradiol and 17beta-trenbolone in bovine serum. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004;802:307-15.
- Watabe Y, Kubo T, Nishikawa T, Fujita T, Kaya K, Hosoya K. Fully automated liquid chromatography-mass spectrometry determination of 17beta-estradiol in river water. *J Chromatogr A* 2006;1120:252-9.
- Center for Drug Evaluation, and Research (CDER). Guidance for Industry: Bioanalytical Method Validation. Rockville, MD: US Department of Health and Human Services, Food and Drug Administration; 2001.
- Proceedings of the International Conference on Harmonization (ICH), Commission of the European Communities; 1996.
- Gibaldi M, Perrier D. Pharmacokinetics. 2nd ed. New York: Marcel Dekker; 1990.

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