Trace Analysis of Ethinyl Estradiol in Casein Diet Using Gas Chromatography with Electron Capture Detection

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Abstract

Ethinyl estradiol (EE_2) is an extremely potent synthetic estrogen and a common component in oral contraceptives. The drug has a well-characterized pharmacological profile and is used as a positive control in toxicological investigations of compounds having estrogenic activity. An analytical method developed for the determination of low µg/kg levels of EE_2 in a casein-based rodent diet is presented. A methanol extract of casein diet is purified for instrumental analysis by a 3-fold solid-phase extraction process. The sample extract is derivatized with pentafluoropropionic anhydride to the pentafluoropropionyl product and analyzed by capillary gas chromatography with electron-capture detection. Recoveries of EE_2 from casein diet fortified at 5, 10, and 50 µg/kg average 88.8% and have a relative standard deviation (%) of 7.2. The method limit of detection in a casein-based diet is 1 µg/kg.

Introduction

Recent studies have reported that estrogen used in combination with a progestin for hormone replacement therapy may increase the risk of breast cancer and coronary heart disease to the extent that the risks may outweigh any benefits of therapy (1). In addition to the synthetic estrogens, many compounds present in the environment (xenoestrogens) can disrupt the endocrine system. However, the link between estrogens and the increased risk of carcinogenesis is still under investigation, and the full impact of chronic human exposure to the large numbers of endocrine-disrupting chemicals in the environment is unknown (2). Therefore, additional toxicology data is needed regarding xenoestrogens.

Ethinyl estradiol (EE_2) (structure 1, Figure 1)is a monosubstituted form of the naturally occurring estrogen 17β-estradiol (structure I, Figure 1). It is one of the most active oral preparations known and is a potent synthetic estrogen. It is widely used in combination (with a progestin) oral contraceptives at doses ranging from 20 to 35 µg daily (3). Mestranol, the only other synthetic estrogen currently in use as a component of oral contraceptives, is converted to EE_2 in vivo (4). EE_2 was first synthesized in 1938 (5) and has since been well characterized. Therefore, EE_2 was selected as a reference compound (positive control) in chronic feeding studies investigating the toxicology of natural phytoestrogens. The drug was mixed at concentrations of 2, 10, and 50 µg/kg with a soy- and alfalfa-free casein diet low in dietary isoflavones. Preferred practice in the conduct of long-term toxicology studies is that the uniformity and stability of the test material in laboratory diet be confirmed by acceptable analytical methods at intervals throughout the study (6). In a few previous studies, EE_2 has been administered to animals through dosed diets (7,8); however, confirmatory analyses either were not performed or were not published. Therefore, no applicable methods with adequate sensitivity were found where EE_2 has been extracted from animal diet and quantitated (9,10). An analytical method was, therefore, required to verify proper dosages, uniformity, and chemical stability of the compound administered in the

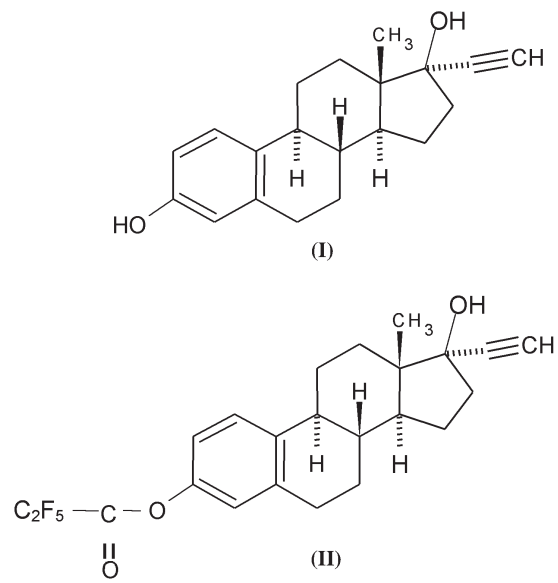

Figure 1. Ethinyl estradiol (I) and its pentafluoropropionyl derivative (II).

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animal's diet at low µg/kg levels. The extraction and assay of test materials from a highly complex matrix such as laboratory diet presents a unique challenge at low µg/kg levels (6).

EE_2 has been determined in pharmaceutical preparations by a variety of methods (11–13). More complex matrices from which EE_2 has been extracted and analyzed include environmental samples (14,15), feces (16), and cervical mucus by gas chromatography (GC)–mass spectrometry (MS) (17). Radioimmunoassay and GC–MS (11–15,18–20) methods have potentially adequate sensitivity; however, both were impractical for the requirements of a long term study because of the associated expense and matrix involvement. Cleanup and analysis of EE_2 by GC and high-performance liquid chromatography were found to lack the necessary sensitivity and recovery (12–14). The method presented here was developed to address EE_2 at fortification levels from 2 to 250 µg/kg. The procedure has been shown to be rugged and appropriate for analysis of EE_2 at low µg/kg levels in a complex matrix of laboratory rodent diet. The procedure also avoided the use of benzene and other suspected harmful solvents.

Experimental

Materials

Reagents

17β-Ethinyl estradiol (purity > 99%) was purchased from Sigma Chemical Co. (St. Louis, MO). Pentafluoropropionic anhydride (PFPA) was obtained from Pierce Chemical Co. (Rockford, IL). Casein-based laboratory diet (5K96, PMI LabDiet) was obtained throughout the study from Purina Mills Int. (Richmond, IN). Solid-phase extraction (SPE) cartridges (Sep-Pak Vac.)—cyano propyl (CN) (5-g), amino propyl (NH_2, 1-g), and silica (Si, 1-g)—were purchased from Waters, (Milford, MA). All solvents (hexane, isopropanol, toluene, and methanol) were resi-analyzed grade and, along with anhydrous sodium sulfate and sodium hydroxide pellets (NaOH), were purchased from JT Baker (Phillipsburg, NJ). Trimethylamine hydrochloride (TMA) was obtained from Fluka Chemika (Buchs, Switzerland). Nujol (mineral oil) was obtained from Alltech Associates (Arlington Heights, IL). Potassium phosphate monobasic (American Chemical Society grade) was obtained from Fisher Scientific Co. (Fair Lawn, NJ).

Prepared reagents

Trimethylamine (0.05M) in toluene was prepared by dissolving 2.0 g of the hydrochloride salt in 5 mL of 5M NaOH, and the free amine was extracted with four 5-mL portions of toluene. Toluene was successively percolated through a plug of anhydrous sodium sulfate (ca., 35-mm diameter × 20-mm thickness). The combined volume was adjusted to 20 mL with toluene. This stock solution (1M) was stored at 5°C. A 1:20 dilution of the stock solution with toluene was prepared as needed to make a 0.05M solution.

Potassium phosphate buffer (0.1M) was prepared using monobasic potassium phosphate in 18 MΩ/cm purified water. The pH was adjusted to 6.0 using 0.1M KOH. The resultant solution was then placed in a separatory funnel and extracted twice with 200 mL toluene to remove trace impurities.

Methods

Feed analyses were performed in triplicate along with a control and triplicate spiked feed containing the appropriate concentrations of EE_2 that was necessary to determine recoveries of the drug. Ten-gram aliquots of dosed animal diet were weighed and transferred to 160-mL polytetrafluoroethylene (PTFE)-lined, screw-capped tubes. Triplicate 10-g aliquots of control animal diet were placed in identical tubes and spiked with 1 mL of either a 100- or 500-ng/mL solution of EE_2 in methanol to make 10 or 50 µg/kg spikes, respectively. Each tube was rolled to disperse the spiking solution throughout the feed sample and allowed to equilibrate for 15 min prior to extraction. Methanol (100 mL) was added to each tube and the contents mixed for 1 h on an Eberbach reciprocating shaker (Eberbach Corp., Ann Arbor, MI). Tubes were then centrifuged at 200 × g for 10 min. An aliquot (50 mL) of each methanol extract was placed into a 200-mL flat-bottomed flask. The methanol was evaporated with a Büchi roto-evaporator using a 60°C water bath. Residues were reconstituted with 10 mL of 0.5% isopropyl alcohol in hexane (0.5%, I/H) and purified by a 3-fold SPE process.

All SPE cartridges were used in the normal phase separation mode. The first SPE phase consisted of 5-g CN cartridges, which were preconditioned with 20 mL of 5% I/H followed by re-equilibration with 40 mL hexane before applying sample residues. Following application of samples, cartridges were rinsed with 10 mL of 0.5% I/H followed by 35 mL 5% I/H. These fractions were discarded. EE_2 was eluted from the cartridges with an additional 35-mL aliquot of 5% I/H, and the eluent was collected in 125-mL flasks. Following roto-evaporation, residues were reconstituted in 2 mL of 1% I/H.

The second SPE phase consisted of 1-g silica cartridges that were preconditioned with 10 mL of 5% I/H then re-equilibrated with 30 mL hexane before applying 2-mL extracts. Cartridges were rinsed with 15 mL of 1% I/H. EE_2 was eluted from the cartridges using 15 mL of 5% I/H and collected in 125-mL flasks. Following roto-evaporation, residues were redissolved in 2 mL of 1% I/H.

The third SPE phase consisted of 1-g amino propyl (NH_2) cartridges, which were preconditioned with 10 mL of 8% I/H then re-equilibrated with 30 mL hexane before applying extracts. Samples were rinsed with 15 mL of 2% I/H followed by 5 mL 8% I/H. EE_2 was collected in the following 20-mL fraction of 8% I/H. Aliquots (1 mL) of Nujol–hexane (5 mg/mL) were added to each flask prior to roto-evaporation. This residue was then used for subsequent derivatization.

Following evaporation to dryness, feed residues were dissolved in 2 mL of 0.05M TMA in toluene and transferred by Pasteur pipet to 8-mL PTFE-lined screw-capped tubes. Appropriate standards in methanol were evaporated with nitrogen, then reconstituted with 2 mL of a 1:1 solution of 0.05M TMA–5 mg/mL Nujol in toluene. Following vortexing, all samples and standards were derivatized with 20 µL of PFPA for 15 min at room temperature. With TMA catalyzing the derivatization reaction, the corresponding pentafluoropropionyl derivative of EE_2 (PFP-EE_2) was formed. The reaction was terminated by the addition of 1 mL of 0.1M potassium phosphate (pH 6.0) buffer followed by vortexing. After centrifugation, the buffer layer was drawn off with a Pasteur pipet and discarded. To prevent hydrolysis of the PFP-EE_2 deriva-

tive, excess unprotonated TMA was removed by washing the samples two more times with 1 mL of buffer. Samples were then centrifuged at 200 × g for 20 min to separate the organic and aqueous phases. While carefully avoiding the aqueous layer, the upper portion of the organic layer was transferred to 1.5-mL Hewlett-Packard GC autosampler vials with PTFE-lined caps (Hewlett-Packard, Palo Alto, CA).

GC analyses

GC analyses were performed on a Hewlett-Packard Model 6890 system, utilizing a capillary column (DB-5; 30-m × 0.25 mm × 0.25-µm film) (J&W Scientific, Folsom, CA) and equipped with an electron-capture detector (ECD). All injections were 2 µL with a split ratio of 30:1. Injection and detector temperatures were 260°C and 325°C, respectively. The column temperature was held at 235°C for 23 min, then increased at 40°C/min to 300°C, and held for 15 min. Helium was used as the carrier gas at a flow rate of 0.6 mL/min, and the detector makeup gas was nitrogen flowing at 60 mL/min. The retention time for PFP-EE_2 was approximately 19.5 min under these conditions. The run sequence was such that each sample or spike injection was bracketed by standard injections.

Results and Discussion

EE_2 was previously shown to be stable in casein diet for at least 16 days. The GC–ECD response for PFP-EE_2 standards from 10 to 250 ng/mL was linear. A least squares fit to the standard data exhibited an R^2 of 0.9987. Responses for EE_2 concentrations up to 625 ng/mL have also been observed to be linear. Recovery of EE_2 from spiked casein diet exceeded 87% at drug concentrations from 5 to 50 µg/kg (Table I). The recovery of EE_2 was acceptable (> 75%) at concentrations up to 300 µg/kg (data not shown). The percent relative standard deviations (%RSDs) obtained for recoveries were 11, 3.6, 6.7, and 8.2 at EE_2 fortification levels of 5, 10, 50, and 250 µg/kg, respectively. Method accuracy and repeatability was demonstrated by results of recovery experiments and the low %RSDs obtained for recovery at all fortification levels in diet. Only at the lowest EE_2 concentration (5 µg/kg) was the %RSD > 10. In addition, a closely related analytical method was used for over 2 years in support of a toxicology study and shown to be robust (21). Overlaid chromatograms of control feed, diet fortified at 10 and 50 µg/kg EE_2, and an EE_2 standard showed minimal interference from the control when analyzed for EE_2 from casein diet (Figure 2). When calculated on the basis of EE_2 concentration, control values at the retention time of EE_2 were consistently less than 1 µg/kg. The limit of detection was determined to be 1 µg/kg, based on three times the standard deviation of background samples. The method is very sensitive considering the highly complex nature of the feed matrix and is also relatively simple, safe, and cost effective to perform.

Table I. Recovery of EE_2 from Casein Diet

EE_2 fortification level (µg/kg)	Number of samples (*n*)	EE_2 concentration recovered	Recovery (%)	RSD (%)
0	3	0.8 ± 0.3	–	–
5	3	4.4 ± 0.5	87.7	11
10	4	8.8 ± 0.3	88.3	3.6
50	4	45.3 ± 3.0	90.5	6.7

The PFP-EE_2 derivative results from the attachment of a single pentafluoropropionyl group (9) at the 3 position (structure II, Figure 1) at room temperature. Elevated reaction temperatures resulted in a mixture of mono- and disubstituted derivatives with attachment of a second pentafluoropropionyl group to the hydroxyl at position 17. Attempts to form only the disubstituted $(PFP)_2$-EE_2 resulted in sample degradation (unpublished data). Therefore, the derivatization reaction should be allowed to occur at room temperature to ensure production of a single derivative.

Split, rather than splitless, injection was used to maintain peak symmetry and sensitivity while minimizing the amount of diet matrix injected. Even with extensive sample cleanup, periodic (~ 100 injections) replacement of the injector sleeve was necessary to maintain sensitivity.

The GC–ECD method was found to be sufficiently sensitive for potential use with other matrices in which EE_2 may accumulate. These may include sewage sludge (22), hair (10), human adipose tissue (5), and bile of aquatic wildlife (15). Bioaccumulation of EE_2 in the adipose tissue of certain wildlife also may be expected because of the drug's extensive distribution and high lipophilicity (3). Possible effects of endocrine disruption upon wildlife include sterility (2,3). The relatively high concentrations of EE_2 found in the environment may be at least partially attributable to the conversion of other steroid pharmaceuticals such as the 3-substitued EE_2 analogs mestranol (4) and quinestrol (23), as well as norethisterone and lynestrenol to EE_2 (15). Also, steroid hormones constitute one of the most highly prescribed class of pharmaceuticals (3).

Estrone is one of the predominant metabolites of EE_2 in humans and animals (5) and is, therefore, an ultimate metabolite of the 3-substitued EE_2 analogs mestranol and quinestrol. Estrone has been found in sludge (37 µg/kg), river sediment (2

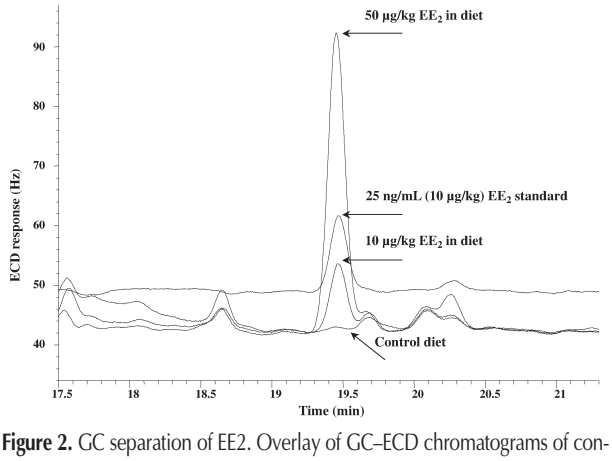

Figure 2. GC separation of EE2. Overlay of GC–ECD chromatograms of control diet, diet fortified with 10 µg/kg EE_2, 10 µg/kg EE_2 standard, and diet fortified with 50 µg/kg EE_2.

µg/kg), sewage effluent water (5 ng/L), and bile of aquatic wildlife (2.5 µg/kg) (15,23). Estrone is a naturally occurring estrogen largely caused by interconversion with 17β-estradiol in the liver (3,24). To investigate the applicability of the method to the analysis of estrone, a series of casein diet samples were spiked with 150 µg/kg estrone and analyzed. Estrone exhibited a greater response to that of EE_2 on an equimolar basis and eluted at 16.1 min but EE_2 eluted at 19.5 min. Recovery of 150 µg/kg estrone from casein diet averaged 93 ± 26%. Expressed on the basis of estrone concentration, the control value at 16.1 min was a negligible 0.1 µg/kg. These data suggest that estrone could be analyzed simulaneously with EE_2 in rodent diet and possibly in a variety of complex matrices. However, extension of this method to the analysis of estrone requires more thorough examination. 17β-Estradiol was not screened for potential analysis by this method.

Detection of EE_2 in cattle feed is possibly a useful regulatory application of the method presented. Most of the beef cattle produced in the United States are administered Food and Drug Administration (FDA) approved growth promoters that are hormonally active. Many of the remaining beef cattle producers take part in the voluntary Non-Hormone Treated Cattle Program (United States Department of Agriculture) to ensure that their product has never been exposed to these substances. Much of this beef is exported to the European Union, which bans all treated imports. EE_2 has not been approved by the FDA for growth promotion (25), and illegal use of EE_2 by European beef producers has been reported (26). Of eight estrogenic growth promoters screened by in vitro methods, EE_2 and 17β-estradiol had the highest activity (27). Also, EE_2 is orally active, but 17β-estradiol is not (3). Therefore, the described method may be applicable to the monitoring of intentionally or accidentally contaminated cattle feeds. Monitoring prior to ingestion may be desirable because the urinary excretion of EE_2 may be artificially reduced up to 5-fold, making this route of noninvasive detection less practical (28).

Conclusion

An accurate and reliable method of analysis for determination of EE_2 in casein-based rodent diet has been described. The method is applicable to the determination of EE_2 at levels from 2 to 250 µg/kg in rodent diet. Because of the need for further studies concerning the many xenoestrogens found in the environment, this method has utility where EE_2 is utilized as a positive control and may have potential use for the screening of cattle feed for accidental or illegal contamination with EE_2.

Safety

PFPA is an acylation derivatization reagent. Acylation reagents should be regarded as potentially toxic and care should be exercised to avoid contact with these reagents. Please review Safety notes for other possible safety concerns and practices.

Acknowledgments

This work was supported by Interagency Agreement 224-93-0001 between the National Institute for Environmental Health Sciences and the United States FDA.

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Manuscript received February 5, 2004;
revision received September 24, 2004.