Short communication

Determination of the antiangiogenesis agent 2-methoxyestradiol in human plasma by liquid chromatography with ultraviolet detection

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Abstract

A high-performance liquid chromatography (HPLC) assay was developed for the quantitative determination of 2-methoxyestradiol (2ME2) in human plasma. Sample pretreatment involved a solid-phase extraction of 1 ml aliquots of plasma with C_{18} micro-columns. Separation was achieved on a Novapak C_{18} column (300 mm × 3.9 mm i.d.; 4 μm PS) at room temperature at an isocratic flow rate of 1 ml/min with 50% acetonitrile in water. Detection was performed at a UV wavelength of 205 nm. Calibration curves were linear in the concentration range of 1–50 ng/ml. The accuracy and precision values obtained from three different sets of quality controls analyzed in replicates of four on four separate occasions ranged from 90.7 to 105.2 and 3.17 to 8.27%, respectively.
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Keyword: 2-Methoxyestradiol

1. Introduction

2-Methoxyestradiol (2ME2) is an endogenous metabolite of estrogen, which is synthesized in vivo by hydroxylation at the 2-position of estradiol, and subsequent catechol-*O*-methyltransferase mediated *O*-methylation (Fig. 1). Plasma concentrations of 2ME2 are in the picomolar range under normal physiological conditions; however, during late pregnancy the values can increase more than 1000-fold [1]. Table 1 provides a more detailed synopsis of the levels of 2-methoxyestradiols in humans in various physiological conditions (adapted from [2]). 2ME2 has been shown to be a potent antiangiogenic and antitumor agent in preclinical models through its apoptotic activity and antimicrotubule activity [3]. 2ME2 is being tested clinically in a number of Phase I and Phase II trials in patients with metastatic breast cancer, prostate cancer and various other solid tumors (reviewed in [3]). Berg et al. developed a radioimmunoassay to quantify 2-methoxyestrogens in

human serum using an ^{125}I-labeled ligand [2]. Robinson et al. [4] described an HPLC with UV detection method for analysis of 2ME2 in extracts from the vitreous humor of rabbit eyes. Zacharia et al. [5] report a yet to be published GC–MS assay for 2ME2 in rat plasma with an LOQ of 0.25 pg/μl. But, none of these methods have previously been used for pharmacokinetic analysis of human plasma in clinical trials. A phase I study of 2ME2 in patients with refractory breast cancer has reported 2ME2 peak concentrations using a GC–MS assay, on Day 1 after dosing, ranging from 5 to 15 nM (1.51–4.53 ng/ml) at the dose of 400 mg administered orally twice a day [6]. Here, we describe the development and validation of an analytical method for the determination of 2ME2 in human plasma samples based on reversed-phase HPLC with UV detection.

2. Experimental

2.1. Chemicals and reagents

2ME2 (HPLC purity, 99.9%) was obtained from Entremed Inc. (Rockville, MD, USA). Methanol (HPLC grade) and

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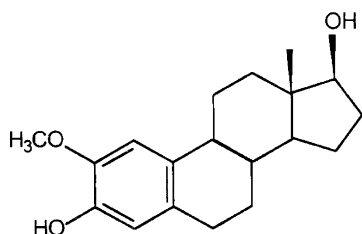*E-mail address:* wdfigg@helix.nih.gov (W.D. Figg).

Fig. 1. Chemical structure of 2-methoxyestradiol (2ME2).

acetonitrile (HPLC grade) were obtained from Mallinckrodt Baker, Inc (Phillipsburg, NJ, USA). Water was filtered and deionized using Milli-Q-UV plus system (Millipore, Milford, MA, USA). Drug free human plasma was obtained from the Blood Bank at the Warren Grant Magnuson Clinical Center (Bethesda, MD, USA).

2.2. Stock solutions and standards

Stock solutions of 2ME2 were made in triplicate by dissolving 10 mg of 2ME2 in 10 ml of methanol resulting in a primary stock solution containing 1 mg/ml of 2ME2; they were stored at −80 °C for up to 4 weeks. Working solutions of 2ME2 were prepared on each analysis day by serial dilutions in methanol from the primary stock solution. The difference in drug concentration in each of the triplicate stock solutions, estimated from the mean peak area following repeat analysis of a dilution of the stock, was determined to be within ±5%.

Six-point calibration containing 2ME2 concentrations of 1, 2, 5, 10, 20 and 50 ng/ml were prepared daily by addition of aliquots the working solutions to 1 ml of drug-free human plasma. Three pools of quality control samples for 2ME2, used for the evaluation of accuracy and precision, were prepared in human plasma at concentrations of 3, 15 and 30 ng/ml. For freeze–thaw stability runs, the aliquots of prepared standards and quality control samples were stored at −80 °C.

Table 1
Concentrations of 2-methoxyestrogens in human serum in various physiological situations

	Median (pg/ml)	Range (pg/ml)	N
Men			
19–58 years	<10.3	<10.3–35.5	22
Women			
Follicular phase	46	18–63	8
Luteal phase	70	31–138	8
Postmenopausal	10	21–76	10
Pregnant			
11–16th week	674	216–1,678	46
37–40th week	3,768	2,035–10,691	34
In labor	3,580	1,353–9,974	41
Newborn			
Cord serum	1,608	575–3,095	41

Abbreviations: N, total number of replicate observations.

2.3. Sample pretreatment

Solid phase extraction was performed using a Vac Elut SPS-24 solid phase extraction chamber and Varian Bondelut C$_{18}$ columns (Harbor City, CA, USA). The columns were conditioned with 2 ml of methanol and then equilibrated using 2 ml of water. One milliliter aliquots of plasma were applied to the column, and then rinsed with 2 ml of 5% methanol (in water). Elution was performed with 2 ml of methanol, which was evaporated to dryness under a continuous stream of air at 40 °C. The extracts were reconstituted in 200 µl of 50% acetonitrile (in water) using vortex-mixing, and 170 µl was injected into the HPLC system.

2.4. Equipment

Chromatography was performed on an HP 1100 system (Agilent Technology, Palo Alto, CA, USA), which included a binary pump, a refrigerated autosampler, a degasser and a photodiode-array detector. Chromatographic separations were performed at ambient temperature on a C$_{18}$ Nova-pak column (300 mm × 3.9 mm i.d.; 4 µm PS; Waters, Milford, MA, USA) using a mobile phase composed of acetonitrile–water (50:50 (v/v)). The mobile phase was delivered isocratically at a flow rate of 1 ml/min, and the effluent was monitored at a UV wavelength of 205 nm. The chromatographic data were collected and analyzed using the Chemstation software (Agilent). Calibration graphs were calculated by least-squares linear regression analysis of the peak area of 2ME2 versus the drug concentration of the nominal standard (x). Calibration curves were fitted by weighted ($1/x^2$) least-squares linear regression analysis. The zero concentration sample (blank) was used to visually verify the purity of the reagents and the lack of other potentially interfering (endogenous) substances, but was not considered for the regression analysis of standards. The goodness-of-fit of various calibration models was evaluated by visual inspection, the correlation coefficient and a lack-of-fit test.

2.5. Validation procedures

Method validation with respect to accuracy and precision was performed according to procedures described in detail elsewhere [7]. The method was validated in terms of linearity of detector response, accuracy, precision, sensitivity, specificity, and freeze–thaw stability. On each validation day, calibration curves were analyzed in duplicate along with quality control samples containing known concentrations of 2ME2. Complete validation runs were performed on four different days using four replicate determinations for each concentration on each day. Statistical analysis was performed using the software package NCSS 2001 (J. Hintze, Number Cruncher Statistical Systems, Kaysville, UT, USA). The extraction recovery was calculated as a percentage by comparing the peak areas of samples prepared at 3 and 50 ng/ml in human

plasma and the mobile phase (50% acetonitrile in water) in triplicate.

2.5.1. Response function

Calibration curves were constructed by least-squares linear regression analysis of peak area ratios of 2ME2 versus the 2ME2 concentration of the nominal standards with or without weighting. To establish the optimal quantification method and weight factor, the correlation coefficient of the fitted equation and the accuracy of back-calculated calibration concentrations were taken into consideration.

2.5.2. Accuracy and precision

Accuracy and precision were determined by analyzing quality control samples with 2ME2 concentrations in the low, mid and high concentration ranges of the calibration curve. Accuracy (DEV) was defined as percent difference between the mean observed concentration and the nominal concentration:

$$DEV(\%) = \frac{[nominal] - [observed]}{[nominal]} \times 100$$

The precision of the assay was assessed by the between-run and within-run precision. The between-groups mean square (MS_{bet}), the within-groups mean square (MS_{wit}), and the grand mean (GM) of the observed concentrations across run days were obtained by one-way analysis of variance (ANOVA) using the run day as classification variable. The between-run precision (BRP) was defined as:

$$BRP(\%) = \frac{\sqrt{(MS_{bet} - MS_{wit})/n}}{GM} \times 100$$

where n represents the number of replicates within each validation run. The within-run precision (WRP) was calculated as:

$$WRP(\%) = \frac{\sqrt{MS_{wit}}}{GM} \times 100$$

2.5.3. Lower limit of quantification

The lower limit of quantitation was defined as the lowest concentration of 2ME2 that could be reliably and reproducibly measured with values for accuracy, between-run precision, and within-run precision of <20%, with concentration determinations performed in at quadruplicate on four separate occasions.

2.5.4. Specificity

Pooled blank plasma samples were used to determine whether endogenous matrix constituents co-eluted with 2ME2. Blank samples were also obtained from five different donors, and were analyzed to determine if there were any interfering peaks around the retention time of 2ME2.

2.5.5. Freeze–thaw stability

The stability of 2ME2 in plasma subjected to three consecutive freeze–thaw cycles was tested by quadruplicate analy-

sis of samples containing 3, 15 and 30 ng/ml of 2ME2. The calculated 2ME2 concentrations were evaluated for accuracy relative to the nominal (spiked) drug concentration.

3. Results and discussion

3.1. Chromatography and detection

Since 2ME2 lacks an efficient functional chromophore, column effluents were analyzed at a UV wavelength of 205 nm (Fig. 2). Typical chromatograms resulting from the HPLC–UV analysis of 1 ml plasma extracts obtained after solid-phase extraction are depicted in Fig. 3, and include a blank plasma, sample (A), a standard spiked at a 2ME2 concentration of 50 ng/ml (B), standard spiked at 2ME2 concentration of 1 ng/ml (C). Fig. 4 depicts a chromatogram obtained from pooled plasma of a number of cancer patients receiving 2ME2. The retention time of 2ME2 was about 6.4 min with an overall run time of 10 min.

3.2. Validation

The lowest and most constant bias across the concentration range investigated was obtained following regression analysis of the data to a linear fit with a weighting factor of $1/x^2$ for the peak area of 2ME2 (data not shown). For each analytical run, a six-point plasma standard curve was constructed, and was shown to be linear over the tested range of 1–50 ng/ml. The mean (±standard deviation) regression equation obtained during the method validation, obtained in duplicates on four separate occasions, showed an intercept of 355 ± 85 and a slope of 21.3 ± 8.5 [Pearson correlation coefficient range (0.9951–0.9779); $n = 4$].

Using this procedure, the lower limit of quantitation was determined to be 1 ng/ml, with a precision of 10.2% and a percent deviation from the nominal standard of
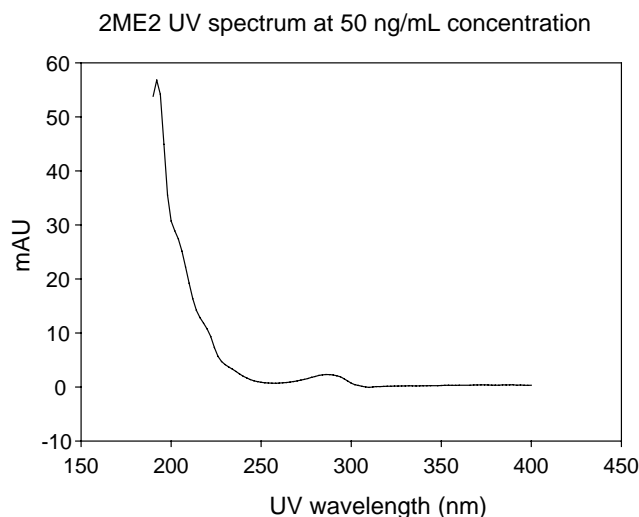

Fig. 2. UV spectrum of 2ME2.

Fig. 4. Chromatogram obtained from pooled plasma of a number of cancer patients receiving 2ME2.

Table 2
Back calculated concentrations from calibration curves

Nominal (ng/ml)	GM (ng/ml)	S.D. (ng/ml)	DEV (%)	R.S.D.	N
1	0.986	0.101	−1.37	0.102	12
2	2.28	0.125	+13.8	0.0551	14
5	5.24	0.351	+4.69	0.0671	14
10	10.6	0.703	+5.53	0.0666	15
20	19.7	1.69	−1.58	0.0860	14
50	48.8	4.15	−2.34	0.0850	13

Abbreviations: GM, grand mean; S.D., standard deviation; DEV, percent deviation from nominal value; R.S.D., relative standard deviation; N, total number of replicate observations (total of all validation runs).

The assay performance data for the determination of independently prepared quality control samples of 2ME2 in plasma are presented in Table 3. The between-run precision and within-run precision ranged from 3.2 to 6.1 and 3.3 to 8.2%, respectively, for the various concentrations tested. At the same concentrations, the values for accuracy were always between −1.6 and +13.8%, which is well within the generally accepted limits for bioanalytical methods.

Blank plasma from different individuals showed a very minor interfering endogenous peak at around the same retention time of 2ME2. However, this endogenous peak did not interfere with the quantification of 2ME2 above the LOQ of 1 ng/ml.
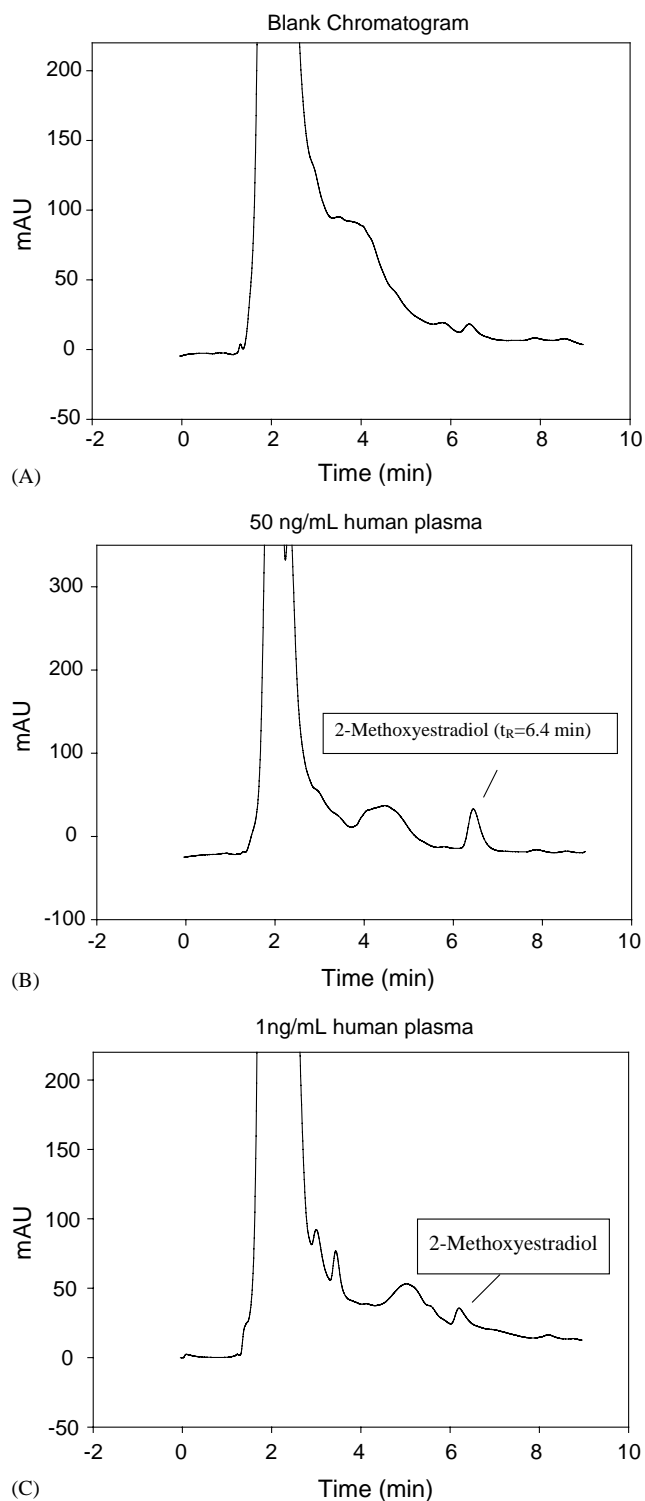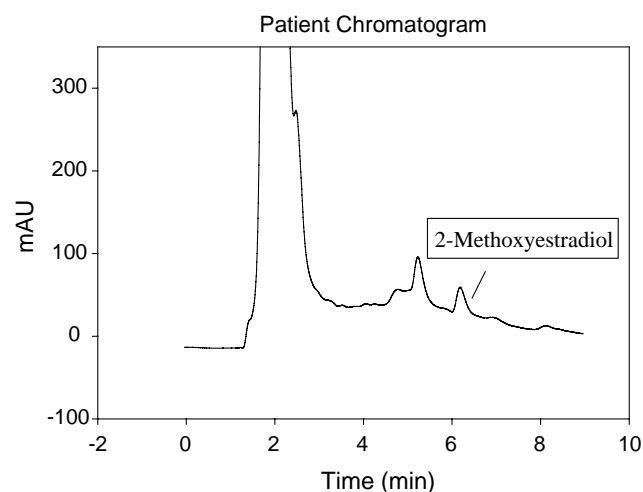
Fig. 3. Typical reverse-phased HPLC–UV chromatograms of: (A) blank human plasma sample, (B) a sample spiked with 2ME2 at a concentration of 50 ng/ml and (C) a sample spiked with 2ME2 at a concentration of 1 ng/ml. The labeled chromatographic peak indicates 2ME2.

−1.4%. Over the entire concentration range of the standard curve, the mean observed percent deviation was between −2.3 and +13.8%, at an imprecision of <10.2% (Table 2).

Table 3
Assessment of accuracy and precision from quality-control samples

Nominal (ng/ml)	GM (ng/ml)	S.D. (ng/ml)	DEV (%)	WRP (%)	BRP (%)	N
3	3.14	0.153	+4.72	3.80	3.17	14
15	15.2	1.00	+1.57	3.32	6.13	15
30	27.2	2.41	−9.31	8.27	3.18	13

Abbreviations: GM, grand mean; S.D., standard deviation; DEV, percent deviation from nominal value; WRP, within-run precision; BRP, between-run precision; N, total number of replicate observations (total of all validation runs).

Three repeated freeze–thawing cycles had no apparent influence on the stability of plasma samples containing 3, 15 or 30 ng/ml. After the third freeze–thaw cycle, 2ME2 plasma concentrations had deviations from the nominal values within the range of -3.5 and $+4.4\%$, irrespective of the tested plasma concentrations as determined by a nonparametric Kruskal–Wallis test ($P > 0.05$). Processed plasma samples were found to be stable at room temperature upon standing in the autosampler tray for at least 18 h (not shown), allowing for overnight analysis of extracted samples. The extraction recovery was found to be 81 and 93% at 3 and 50 ng/ml, respectively.

4. Conclusion

To date, only one method utilizing radioimmunoassay has been described for analysis of 2-methoxyestradiols in human serum, which although more sensitive (LOQ = 10.3 pg/0.5 ml) is marred by cross-reactivity with similar estrogenic compounds i.e. 2-methoxyestrone, 2-methoxyestriol and 2-hydroxyestrone. Here, we describe a simple and rapid assay method that was developed and validated for the determination of 2ME2 in human plasma. The performance criteria for sensitivity, accuracy, precision, linearity, stability, and specificity were acceptable, indicating that the method can be used for determination of 2ME2 in plasma samples obtained from patients treated with the drug. The method is currently being used to study the pharmacokinetic profile of 2ME2 in patients diagnosed with cancer.

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