Plasma protein binding of the investigational anticancer agent 2-methoxyestradiol

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2-Methoxyestradiol (2ME2) is an endogenously produced metabolite of estradiol currently being tested in phase I and II clinical trials as an anticancer agent. Here, we examined the role of protein binding as a possible determinant of the pharmacokinetic behavior of 2ME2. The distribution of 2ME2 in plasma was studied *in vitro* using plasma from healthy human volunteers and *ex vivo* using plasma from patients with cancer receiving the drug orally. The equilibrium dialysis method used to characterize plasma protein binding of 2ME2 utilized a tracer amount of [^{3}H]-2-methoxyestradiol on a 96-well microdialysis plate with a 5-kDa cutoff membrane and 250 µl of plasma. The time to equilibrium was approximately 24 h and the mean unbound fraction of 2ME2 (f_u) over the observed concentration range in plasma of patients receiving 2ME2 orally was 0.019 ± 0.0043. The mean f_u was 0.027 ± 0.0019 in plasma of healthy human volunteers. The binding was concentration independent, indicating a low-affinity, possibly nonspecific and nonsaturable process. The binding was also unaffected by the presence of 2-methoxyestrone, one of the major metabolites of 2ME2. 2ME2 was found to bind in decreasing order to plasma > albumin > α_1-acid glycoprotein > sex-hormone-binding globulin. Plasma concentration–time profiles of total 2ME2 and unbound 2ME2 concentrations in a patient with cancer receiving 2ME2 as a single oral dose were parallel to each other. Thus, indicating that plasma protein binding is not an important consideration in pharmacokinetic monitoring of 2ME2. *Anti-Cancer Drugs* 17:977–983 © 2006 Lippincott Williams & Wilkins.

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Introduction

2-Methoxyestradiol (2ME2) is an endogenous metabolite of estrogen, which is produced *in vivo* through a hydroxylation at the C2-position of 17β-estradiol by cytochrome P450 enzymes and subsequent catechol-*O*-methyltransferase-mediated *O*-methylation. Plasma concentrations of 2ME2 are in the picomolar range under normal physiological conditions; however, during late pregnancy these values are increased 1000-fold [1]. 2ME2 has been shown to inhibit tumor proliferation through its antiangiogenic and pro-apoptotic activities, and it is currently being evaluated as an anticancer agent in clinical trials involving patients with solid tumors (reviewed in [2]).

A preliminary pharmacokinetic evaluation of 2ME2 given orally to cancer patients has shown a relatively long terminal half-life of 2ME2 in plasma of approximately 1–2 days, although overall gastrointestinal absorption appears to be low [3]. In contrast, the half-life of 2ME2 in male Sprague–Dawley rats receiving 2ME2 as an intravenous bolus has been reported to be only about 20 min [4]. The basis for this long half-life in humans is unknown, but may possibly be related to species differences in enterohepatic recirculation processes. A variety of other factors, however, may influence the prolonged circulation of 2ME2 in humans, including binding of the compound to plasma proteins. Indeed, drugs with high affinity for plasma proteins often demonstrate a relatively slow distribution and elimination of drug from the central compartment, which may prolong the apparent half-life. Furthermore, various estrogens have been shown to bind extensively to albumin and the sex-hormone-binding globulin (SHBG). For example, Dunn *et al.* [5] have reported that in human females 37% of estradiol is SHBG bound, 61% is albumin bound and 1.8% is unbound, while in human males 78% of estradiol is albumin bound, 20% is SHBG bound and 2.3% is unbound.

The purpose of this study was to characterize the binding properties of 2ME2 to human plasma and individual proteins using a novel microequilibrium dialysis method that might aid in understanding the clinical pharmacokinetic properties of this compound.

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Methods

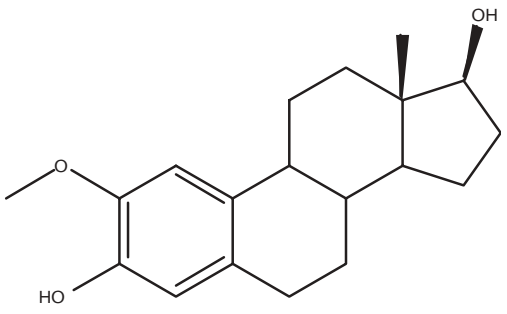
Chemicals and reagents

2ME2 (chromatographic purity: 99.99%) (Fig. 1) and [^{3}H]-2ME2 (specific activity: 5 Ci/mmol) were kindly supplied by Entremed (Rockville, Maryland, USA). High-performance liquid chromatography-grade methanol, ethanol and acetonitrile were obtained from J.T. Baker (Phillipsburg, New Jersey, USA). Deionized water was generated with a Hydro-Reverse Osmosis system (Durham, North Carolina, USA) connected to a Milli-Q UV Plus purifying system (Marlborough, Massachusetts, USA). Bio-Safe II scintillation fluid was obtained from Research Products International (Mount Prospect, Illinois, USA). Albumin, α_1-acid glycoprotein (AAG), SHBG and 2-methoxyestrone (2ME1) were obtained from Sigma-Aldrich (St Louis, Missouri, USA). Other chemicals were of reagent grade or better. Pure protein solutions at respective physiological concentrations were prepared in 0.01 mol/l phosphate buffer (pH 7.4). The stock solutions of all test substances were prepared in ethanol. Drug-free human whole blood was obtained from patients with cancer, and the plasma fraction was separated by centrifugation (3000 *g* for 5 min at 37°C) and frozen within 1 h after collection. Drug-free heparinized human plasma from healthy volunteers was obtained from the National Institutes of Health Clinical Center Blood Bank (Bethesda, Maryland, USA).

Equilibrium dialysis

Equilibrium dialysis was performed on a plate rotator (model no. 74-2334; Harvard Apparatus, Holliston, Massachusetts, USA) at 37°C in a humidified atmosphere of 5% CO_2 using 96-well microdialysis plates (Harvard Apparatus) [6]. The dialysis compartments in each well were separated by a regenerated cellulose membrane with a 5-kDa cutoff. Experiments were carried out with 250-µl aliquots of plasma containing a tracer amount of [^{3}H]-2ME2 (5 µl of 0.02 mCi/ml) against an equal volume of 0.01 mol/l phosphate buffer (pH 7.4). Drug concentrations in 125-µl aliquots of both compartments were measured by liquid scintillation counting for 1 min after the addition of Bio-Safe II scintillation fluid on a Model LS6000IC counter (Beckman Instruments, Columbia, Maryland, USA).

Fig. 1



Chemical structure of 2-methoxyestradiol.

Method validation

The precision of the developed assay was assessed by calculation of 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 a one-way analysis of variance (ANOVA), using the run day as classification variable. The between-run precision (BRP) was defined as:

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

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

$$\mathrm{WRP} = \frac{\sqrt{\mathrm{MS_{wit}}}}{\mathrm{GM}} \times 100\%.$$

The precision was assessed by analyzing quadruplicate samples prepared from four different plasma sources in quadruplicate on four separate occasions. Within-assay and between-assay precision estimates were obtained by a one-way ANOVA, and reported as relative standard deviation.

To evaluate the specificity of the procedure and check for displacement effect of 2ME1 on the protein binding of 2ME2, blank human plasma was spiked with a tracer amount of [^{3}H]-2ME2 (5 µl of 0.02 mCi/ml) and 2ME1 at concentrations of 10, 100, 500 or 800 ng/ml, or 1, 10 or 40 µg/ml, and analyzed in quadruplicate for changes in the fraction of unbound drug (f_u).

The stability of [^{3}H]-2ME2 at 37°C was assessed in human plasma and 0.01 mol/l phosphate buffer [pH 7.4; phosphate-buffered saline (PBS)] by comparing, in quadruplicate, the liquid scintillation counts obtained after 24 h with freshly prepared samples. The stability of unlabeled 2ME2 in plasma at 37°C was analyzed by comparing the chromatographic peak area in quadruplicates of a 1-ng/ml solution of 2ME2 in plasma kept for 24 h using an analytical method based on liquid chromatography with tandem mass spectrometric detection, as described previously [7]. The freeze–thaw stability of [^{3}H]-2ME2 was established by comparing, in quadruplicate, the liquid scintillation counts obtained by plasma samples spiked with [^{3}H]-2ME2 after one freeze–thaw cycle, with freshly prepared plasma samples containing an equal amount of [^{3}H]-2ME2. The freeze–thaw stability of unlabeled 2ME2 in human plasma has been described elsewhere [7]. Samples were considered stable if the difference between peak areas (for 2ME2 in

plasma) or liquid scintillation counts (for [^{3}H]-2ME2 in plasma or buffer) was within ± 20% of the nominal spiked value.

In order to calculate the recovery, two groups of samples (groups A and B) were prepared in replicates of eight. In group A, plasma and 0.01 mol/l phosphate buffer (pH 7.4) from both sides of the equilibrium dialysis well (around 500 µl) were completely emptied into the scintillation counting fluid. Group B comprised freshly prepared samples with 250 µl of PBS and 250 µl of plasma with 5 µl of [^{3}H]-2ME2 (0.02 mCi/ml). Recovery was calculated using the following formula and expressed as percentage:

$$\text{Percentage recovery} = \frac{\text{scintillation count group B}}{\text{scintillation count group A}} \times 100.$$

Time to equilibrium and saturation of binding

The time course of equilibrium was assessed in quadruplicate at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 18, 22, 24, 30 and 48 h after the start of the experiment. The fraction of unbound drug (f_u) was also determined in plasma samples from healthy human volunteers over the anticipated clinically relevant concentration range of 2ME2 (i.e. 0, 1, 5, 10, 15, 20 and 30 ng/ml) and at concentrations higher than that at 40, 100 and 400 ng/ml to test saturability of plasma protein binding.

Binding to purified human proteins

The binding of 2ME2 to purified human proteins was studied by testing various protein concentrations in triplicate in 0.01 mol/l phosphate buffer (pH 7.4). Albumin binding was studied within the concentration range of 1–5 g/dl (physiological range: 3.5–4.5 g/dl), AAG binding was studied within the concentration range of 0.01–0.25 g/dl (physiological range: 0.1–0.25 g/dl) and SHBG binding was studied within the concentration range of 10–200 nmol/l (physiological range: 13–130 nmol/l).

Estimation of binding parameters

The drug concentration ratio in the buffer and plasma or protein solution after dialysis was calculated for each paired observation, and was taken as an estimate of f_u. The bound drug fraction (f_{bd}) was calculated as:

$$f_{bd} = (1 - f_u) \times 100\%.$$

Estimation of binding parameters to individual proteins was determined using the data obtained from buffer solutions of purified human proteins. The drug concentration ratio in the buffer protein solution after dialysis was calculated for each paired observation and was taken as an estimate of the unbound drug fraction (f_u). The bound drug fraction (f_{bd}) was calculated as $f_{bd} = (1 - f_u)$. The total binding constant (nK) for the drug interactions with isolated proteins (albumin, AAG and SHBG) was calculated using the following equation, assuming a single class of nonsaturable binding sites and no allosteric effects:

$$C_b = nK \times P \times C_u$$

$$f_u = \frac{1}{1 + nK \times P},$$

where P is the protein concentration, C_u is the unbound drug concentration, C_b is the bound drug concentration and nK (product of number of binding sites by affinity constant) denotes the total binding constant. Here, $nK \times P$ is the slope of the regression line, and C_{bd}, C_u and P are expressed as molar concentrations (mol/l).

$$f_b = 1 - f_u = 1 - \frac{1}{1 + nK \times P} = \frac{nKP}{1 + nK \times P}$$

$$f_u / f_b = \frac{1}{nK \times P}.$$

Rearrangement of these equations indicates:

$$nK = f_u / (f_b \times P).$$

Here, n represents the number of saturable binding sites per mole of protein, P is the molar concentration of protein binding, K is the association constant and nK is the contribution constant of nonspecific, nonsaturable binding on one site (per molar concentration of protein).

Patients and treatment

Plasma samples from five patients with cancer, who received single oral doses of 2ME2 orally, were used to determine f_u of 2ME2 *in vivo*. From each patient, serial whole-blood samples were obtained during the first course of treatment at the following time points: (1) immediately before drug administration (pre-dose), and (2) at 0.5, 1.5, 2, 3, 4, 5, 6, 9, 12, 24, 38 and 50 h after the first drug administration. All samples were immediately placed in an ice-water bath, centrifuged within 30 min of collection at 1000 *g* for 10 min at 4°C and stored at or below −70°C until analysis. The clinical protocol was approved by the Institutional Review Board (National Cancer Institute, Bethesda, Maryland, USA) and all patients provided written informed consent.

Measurement of total drug concentrations

Total 2ME2 concentrations (i.e. the total of C_u and C_{bd}) were determined using a validated analytical method based on liquid chromatography coupled with tandem mass spectrometric detection [5].

Measurement of unbound drug concentrations

The f_u of 2ME2 in each individual patient plasma sample was determined using equilibrium dialysis as described above for spiked samples. The C_u was calculated from the

f_u and the total drug concentration in plasma (C_p), as follows:

$$C_u = f_u \times C_p.$$

Pharmacokinetic analysis

Estimates of pharmacokinetic parameters for total and unbound 2ME2 in plasma were derived from individual concentration–time data sets by noncompartmental analysis using the software package WinNonLin version 4.0 (Pharsight, Mountain View, California, USA). The area under the plasma concentration–time curve (AUC) was calculated using the linear trapezoidal method from time zero to the time of the final quantifiable concentration (AUC_{tf}). The AUC was extrapolated to infinity (AUC_{inf}) by dividing the last measured concentration by the rate constant of the terminal phase (k), determined by log-linear regression analysis. The apparent oral clearance of 2ME2 (CL/F) was calculated by dividing the administered dose by the observed AUC_{inf} and the terminal half-life was calculated as $\ln 2/k$ (Table 1).

Statistical considerations

All experiments were performed at least in triplicate on at least three separate occasions and statistical analyses were carried out using NCSS version 2001 (J.L. Hintze, Kaysville, Utah, USA). The effects of 2ME2 concentration and protein concentrations on drug binding were estimated by a one-way ANOVA. All data are presented as mean values ± standard deviation, unless stated otherwise, and for all tests the *a priori* cutoff for statistical significance was taken at a P value less than 0.05.

Results

Validation of dialysis method for 2-methoxyestradiol

Preliminary experiments revealed that equilibrium was attained around 24 h (Fig. 2); these data were modeled using Graphpad Prism version 2.0. The mean coefficient of variation of all sample values was less than 20%, assuring high discriminatory power in the detection of changes in the fraction unbound 2ME2 in patient samples. With the final method, the within-run and between-run variabilities of the developed method were 7.1 and 8.5%, respectively. [^{3}H]-2ME2 was found to be stable in plasma and PBS at 37°C for at least 24 h, whereas unlabeled 2ME2 was also found to be stable in plasma at 37°C for 24 h. [^{3}H]-2ME2 was found to be stable in plasma after one freeze–thaw cycle and the overall recovery for [^{3}H]-2ME2 was found to be 92%.

Table 1 Summary of pharmacokinetic parameters of 2ME2[a]

Parameter	Unbound 2 ME2	Total 2ME2
C_{max} (ng/ml)	0.2	18.6
T_{max} (h)	0.5	0.5
AUC (ng × h/ml)	6.0	704.23
CL/F (l/h/m^2)	266.6	2.272
V/F (l)	4596	62.77
$T_{1/2}$ (h)	17.24	27.63

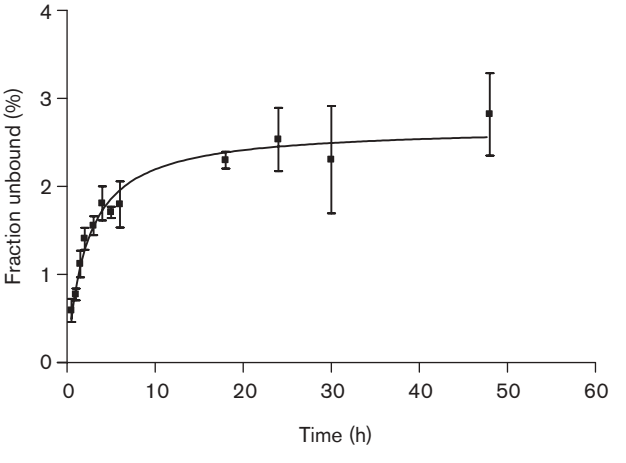
[a]Data were obtained from one patient who received 2ME2 orally at a dose of 1600 mg.
2ME2, 2-methoxyestradiol; C_{max}, peak plasma concentration; T_{max}, time to peak concentration; AUC, area under the plasma concentration–time curve; CL/F, apparent oral clearance; $T_{1/2}$, half-life of the terminal phase

In-vitro binding interactions with 2-methoxyestradiol

2ME2 was found to bind highly to human plasma (mean, 97.4 ± 0.22%), with a mean free-drug fraction of 2.7 ± 0.19%. No significant source difference was observed in the fraction unbound 2ME2 when plasma was used from different healthy individuals (mean f_u, 2.68; $P > 0.05$; one-way ANOVA). The fraction unbound 2ME2 obtained in previously frozen plasma from healthy volunteers was found to be slightly higher than that observed in the plasma from six cancer patients (mean f_u, 2.6 versus 1.9%; $P = 0.045$).

At clinically relevant concentrations of 2ME2 (1–30 ng/ml), the binding was concentration independent ($P > 0.05$), indicating a low-affinity, possibly nonspecific and nonsaturable process. 2ME2 binding to albumin (1–5 g/dl), AAG (0.1–0.25 mg/dl) and SHBG (1–20 µg/ml), however, was found to be concentration dependent, with the fraction of unbound drug decreasing with an increase in protein concentration ($P < 0.05$; one-way ANOVA). The contribution constant of nonspecific binding (nK) of 2ME2 to albumin was 4.29 ($\pm$ 0.420) × 10^4/mol/l, 4.41 ($\pm$ 0.139) × 10^4/mol/l for AAG and 1.5 × 10^6 ($\pm$ 2.6) × 10^5/mol/l for SHBG. Table 2 provides a detailed synopsis of the effect of concentration of different proteins on the fraction unbound 2ME2. Figure 3 provides a histogram comparing the binding of 2ME2 with human plasma, albumin, AAG and SHBG.

Fig. 2



Time course to reach equilibrium for the fraction unbound 2-methoxyestradiol (2ME2). Data are presented as means of individual observations (symbols) and a predicted model fit according to a modified Hill function ($r^2 = 0.85$) with standard deviations (error bars).

Table 2 Binding of 2ME2 to human plasma proteins

Protein	Concentration	Mean f_u (%)	*n*
Albumin	50 mg/ml	3.05	3
Albumin	40 mg/ml	3.75	3
Albumin	25 mg/ml	5.3	3
Albumin	10 mg/ml	10.5	3
AAG	2.5 mg/ml	27.6	3
AAG	1 mg/ml	48.8	3
AAG	0.5 μg/ml	63.4	3
AAG	0.1 mg/ml	81.5	3
SHBG	20 μg/ml	73.3	2
SHBG	5 μg/ml	85.5	3
SHBG	1 μg/ml	92.6	3

2ME2, 2-methoxyestradiol; AAG, α_1-acid glycoprotein; SHBG, sex-hormone-binding globulin; f_u, fraction unbound 2ME2; *n*, number of replicate observations.

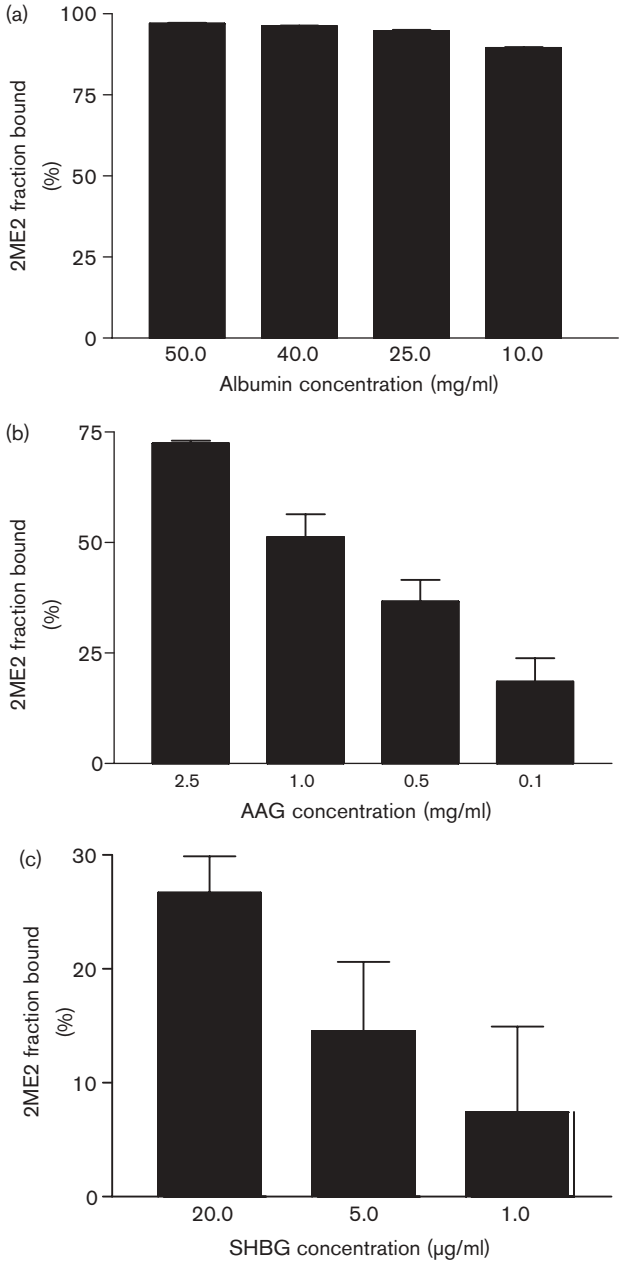
Displacement interactions on binding sites

Concentrations of the 2ME2 metabolite 2ME1 measured in plasma are approximately 100- to 200-fold higher than 2ME2 concentrations after administration of oral 2ME2 in humans [8]. Hence, we examined the effect of 2ME1 on the binding of 2ME2. No change was observed in the fraction unbound 2ME2 in the presence of 2ME1 over a range of 10 ng/ml to 10 μg/ml ($P = 0.78$, *t*-test).

Clinical pharmacokinetics of unbound 2-methoxyestradiol

The equilibrium dialysis method was next applied to define the concentration–time profiles of total and unbound 2ME2 in five patients with cancer who received a single oral dose of 2ME2. One patient received 2ME2 at a dose of 800 mg, two patients received 2ME2 at a dose of 1600 mg and two patients received 2ME2 at a dose of 2200 mg. Total 2ME2 concentrations detected in plasma of these patients were very low, and ranged between 1.1 and 18.6 ng/ml. A combined concentration–time profile of total and unbound 2ME2 as measured in one representative patient who received a single oral dose of 1600 mg is provided in Fig. 4(a). The fraction of unbound 2ME2 was calculated for all five patients of found to be constant over time at all the pharmacokinetic time points (Fig. 4b). Table 1 provides a comparison of the pharmacokinetic parameters obtained in this patient for unbound and total 2ME2 concentrations, and confirm the high binding of 2ME2 *in vivo*, and the parallel decline in concentrations for unbound and total 2ME2.

Fig. 3

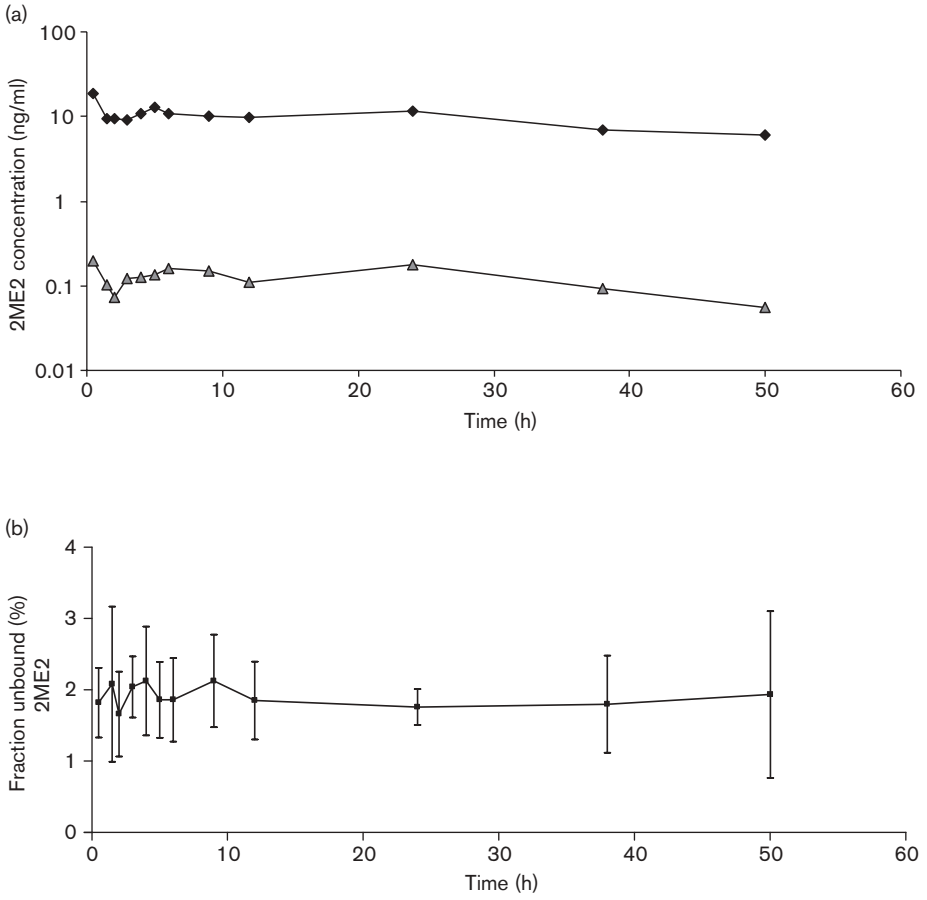


Binding of 2-methoxyestradiol (2ME2) to human plasma proteins. Data are presented as mean values (bars) ± standard deviation (error bars). (a) Albumin; (b) AAG, α_1-acid glycoprotein; (c) LDL, SHBG, sex-hormone-binding globulin.

Discussion

In the present study, we have described the in-vitro and ex-vivo plasma protein binding of 2ME2. The binding of 2ME2 to human plasma *in vitro* was approximately 98% and independent of drug concentration over the clinically relevant range. When binding studies were extended to individual proteins, it was found that 2ME2 binds extensively to human albumin, AAG and SHBG. The association constant for binding was 4.29 (± 0.420) $\times 10^4$/mol/l for albumin and 4.41 (± 0.139) $\times 10^4$/mol/l for AAG, suggesting that both proteins bind to 2ME2 with similar affinity. The association constant for binding for SHBG was 1.5 (± 2.6 $\times 10^5$) $\times 10^6$/mol/l. A slight but statistically significant increase was noted in the fraction of unbound 2ME2 observed in plasma obtained from healthy volunteers compared with plasma from cancer patients. This type of effect of disease state on drug binding

Fig. 4



(a) Plasma concentration–time profiles of total 2-methoxyestradiol (2ME2) (ng/ml) and unbound 2ME2 (ng/ml) in a representative patient who received 2ME2 orally at a dose of 1600 mg. Total and unbound concentrations of 2ME2 are represented by squares and triangles, respectively. (b) The mean (symbol) ± standard deviation (error bars) fraction of unbound (f_u) 2ME2 in plasma versus time profiles. Data were obtained from five patients with cancer who were given a single oral dose of 2ME2.

has been described previously for various agents, including fluconazole [9], methadone [10] and propranolol [10].

We found that the 2ME2 metabolite, 2ME1, had no statistically significant effect on the binding of 2ME2 to human plasma. Previous investigation has demonstrated that 2ME2 is extensively metabolized into 2ME1 in humans. 2ME1, however, seems to be devoid of antiproliferative and antiangiogenic activity both in in-vitro models [11] and in rodent tumor models [12], indicating that formation of 2ME1 is an efficient detoxification pathway. The lack of effect of 2ME1 on the binding properties of 2ME2 suggests that the pharmacological ramification of this metabolic pathway with respect to a potential change in the systemic exposure to unbound 2ME2 is relatively limited.

Although there was very low interindividual variability in the fraction of unbound 2ME2 in the plasma of cancer patients at the 2ME2 dose levels tested, there was very high interindividual variability in the total 2ME2 concentrations measured. Consistent with the in-vitro data, almost 98% of drug was bound within the circulation without any trend over time. Although there was no change in the fraction of unbound drug over the therapeutically achieved concentrations of 2ME2, there were significant changes in the fraction of unbound drug with the protein concentration when tested in the presence of albumin, AAG and SHBG. Variability in protein binding, partly due to variability in albumin and AAG concentrations, however, contributed to the variability in total 2ME2 exposure, but did not substantially affect the unbound drug exposure, and thus a drastic effect of changes in protein binding on the pharmacological response to 2ME2 may be unlikely. Therefore,

protein binding does not seem to be an important consideration in pharmacokinetic monitoring for 2ME2 in cancer patients. It should be pointed out, however, that the binding of 2ME2 to plasma proteins should be taken into consideration when attempting to extrapolate data obtained in tumor-bearing animals and in in-vitro studies to the clinical situation, as described previously for other angiogenesis inhibitors such as suramin, carboxyamidotriazole and 7-hydroxystaurosporine (UCN-01) [13].

In conclusion, a reliable and reproducible equilibrium dialysis method for the determination of the fraction of unbound 2ME2 in plasma was developed and validated. The extent of binding of 2ME2 to albumin and AAG was higher than estradiol, while the extent of binding to SHBG was similar to that reported previously for estradiol [3]. 2ME2 was found to bind with a high degree of affinity to several human plasma proteins, including albumin and AAG. Further experiments in animals and patients should be performed to investigate associations between total and unbound drug exposure and the efficacy and toxicity of 2ME2.

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