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Analysis of clomiphene isomers in human plasma and detection of metabolites using reversed-phase chromatography and fluorescence detection

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Abstract: A method is described for the quantitative clinical analysis of plasma concentrations of the E and Z isomers of clomiphene, which is used in the induction of ovulation. The isomers of clomiphene, in addition to metabolites, are extracted from plasma with *tert*-butyl methyl ether (MTB). The MTB layer is dried, reconstituted and an aliquot subjected to chromatography. The drug and metabolites are separated by reversed-phase high-performance liquid chromatography. The eluent is fed into a knitted or braided, cylindrical reaction coil made of Teflon, into which is inserted a low-energy mercury lamp. This results in a photoinduced stilbene-to-phenanthrene oxidation yielding highly fluorescent analytes; this provides excellent sensitivity for the quantitation of the intact drug isomers and the detection of presently uncharacterized metabolites. Use of the reversed-phase chromatographic mode results in elution of the polar metabolites prior to the intact drug isomers. A combination of reversed-phase chromatography and an in-line post-column reaction coil results in a sensitive method that is more reliable and rapid than those previously reported and is applicable to the routine analysis of clinical samples. The method has been applied to individual isomers of clomiphene in plasma at concentrations of 0.06–600 ng/ml.

Keywords: *Clomiphene isomers; reversed-phase high-performance liquid chromatography; post-column reaction coil.*

Introduction

Clomiphene citrate is a synthetic oestrogen agonist–antagonist used to induce ovulation in anovulatory patients. The therapeutic activity of the drug in human patients is generally considered to result from its anti-oestrogenic effect. Clomiphene inhibits the negative feedback of endogenous oestrogens; this results in elevated concentrations of pituitary gonadotropins, which in turn cause follicular growth and ovulation. However, both animal and human studies show that clomiphene also possesses oestrogenic activity. These multiple effects have led to confusion about the mechanism of action of the drug [1].

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Although clomiphene has been used therapeutically for approximately two decades, little is known of either its pharmacokinetic characteristics or its metabolism. Clomiphene is administered orally as a mixture of E and Z isomers; the Z isomer content is not less than 30% and not greater than 50% [2]. Consequently its pharmacokinetic characterization involves two distinct drug entities. A study in humans using ^{14}C-labelled clomiphene, which is not analytically specific in respect of the two isomers, showed that 51% of the label was excreted in the faeces during 5 days and that the label was found in the faeces up to 6 weeks following dosing [3]. In human plasma samples examined 2–3 h following administration of a single 100-mg dose, the mean concentrations of the E and Z isomers were 23 and 43 ng/ml, respectively [4].

The pharmacokinetic behaviour of a structurally similar compound, tamoxifen, has been described. Tamoxifen is excreted almost exclusively in the faeces and has an apparent half-life of β-phase elimination of greater than 7 days. This compound is suspected to undergo enterohepatic recirculation [5]. Similar pharmacokinetics are expected for the individual isomers of clomiphene.

For pharmacokinetic studies in animals and humans a simple, sensitive and reliable analytical method for determining the E and Z isomers of clomiphene in plasma is desirable. The present paper reports such a method which combines several existing techniques into an integrated method that permits the detection of low levels of each isomer in plasma. The separation of the E and Z isomers is accomplished by reversed-phase high-performance liquid chromatography (HPLC). The reversed-phase mode offers the advantages of greater dependability and reproducibility in comparison with the normal-phase method previously reported [4]. In addition, the reversed-phase mode results in elution of metabolites prior to the clomiphene isomers, thereby eliminating inter-injection wash-out of the highly retained metabolites in the normal-phase mode [4].

The chemical reaction on which the method is based is a photochemically induced stilbene-to-phenanthrene oxidation that results in the formation of highly fluorescent analytes. Application of this reaction has been reported for diethylstilboestrol and tamoxifen [6], as well as for clomiphene [4]. The method described in the present work involves an in-line post-column conversion of clomiphene to fluorescent analytes and is similar to the procedure previously reported for the drug [4]. However, in the present method a knitted or braided reaction coil is used in which the photochemical conversion takes place [7, 8].

Experimental

Materials

HPLC grade methanol purchased from Fisher Scientific Co. was used as received. Water, first treated by reverse osmosis, was distilled in an all-glass apparatus. *Tert*-butyl methyl ether (MTB) (Aldrich Chemical Co., Inc.) was double distilled before use. Reagent grade phosphoric acid (J.T. Baker Chemical Co.) and triethylamine (MCB Manufacturing Chemists Inc.) were used as received. Clomiphene citrate was purchased from Sigma Chemical Co. and was used without further treatment.

Equipment

The chromatographic system included a solvent delivery system (Waters Associates, Inc. Model 6000A) and a syringe loading injection valve (Rheodyne Model 7125). The detection system comprised a fluorescence spectrophotometer (Perkin–Elmer Model

650-10S) fitted with an 18-μl micro-flow cell unit (Perkin–Elmer). The excitation monochromator was set at 255 nm and the emission monochromator at 378 nm. Both excitation and emission slit widths were 15 nm. Chromatograms were recorded with a 10 mV strip-chart recorder (Omniscribe Model B5117-1, Houston Instruments). A UV absorbance detector (Model 440, Waters Associates, Inc.) was used in some reaction coil optimization and characterization experiments.

The photochemical reaction coil was constructed of Teflon (PTFE) tubing (Cope Plastics, Topeka, KS), of inside diameter 0.79 mm and outside diameter 1.61 mm. The reaction coil was knitted in a manner similar to that described in a reported method [7]. In contrast to the reported method, where the knitter used a 3- or 4- peg 'strickliesel', which resulted in a coil with a dense rope-like configuration, the coil used in the present work was knitted around 16 pegs, which resulted in a hollow cylinder. The resulting cylinder, constructed from 18 m of Teflon tubing, was 80 mm in diameter and 120 mm high. The photochemical induction source was a low-pressure mercury lamp (General Electric Model G4T4.1), which was inserted into the centre of the knitted teflon cylinder so that the position of the source lamp was 20 mm from the inside surface of the knitted Teflon cylinder. The coil and lamp were housed in a wooden chamber that was positioned in-line between the column outlet and the detector inlet. Figure 1 is a schematic diagram of the equipment. The reactor does not generate ozone and does not require any special cooling equipment.

Chromatographic conditions

Separation of the E and Z isomers of clomiphene was accomplished on a 150 × 4.6 mm i.d. column packed with 5-μm spherical octyl dimethyl silyl bonded phase (LC-8 Supelco). The mobile phase comprised methanol–water (80:20, v/v) with 2.30 ml of phosphoric acid and 10.0 μl of triethylamine per l. The flow rate was 1.5 ml/min, and temperature was ambient.

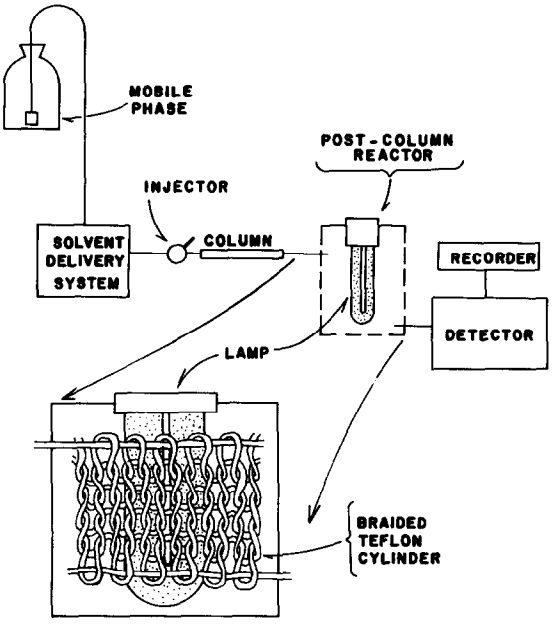

Figure 1
A schematic diagram of the chromatographic and detection systems.

Extraction procedure

The Z and E isomers of clomiphene were extracted from samples (3.0 ml) of both spiked human plasma and standard aqueous solutions prepared to contain individual isomer concentrations in the range of 0.06–600 ng/ml. The 3.0-ml samples were transferred by pipette into 20-ml screw-top centrifuge tubes, the tops of which were fitted with Teflon (PTFE) liners. Samples (12 ml) of MTB were added and the tubes were rotated end-over-end at 50 rpm for 30 min. The sample tubes were then centrifuged for 5 min. Volumes (10 ml) of the MTB layer were transferred to test tubes, and the contents were evaporated to dryness under a nitrogen stream. At the time of analysis, the dried residues contained in the test tubes were reconstituted with 0.5 ml of methanol–water (80:20, v/v). Volumes of 100-μl of each solution were injected into the chromatograph.

Results and Discussion

Chromatography

A chromatogram obtained from a spiked, extracted human plasma sample is shown in Fig. 2. Calculation of apparent capacity factors, without accounting for the reaction coil residence time, resulted in $k'_{\text{apparent}} = 2.3$ and $k'_{\text{apparent}} = 1.9$ for the Z and E isomers, respectively (separation factor, $\alpha = 1.2$). Removal of the reaction coil residence time

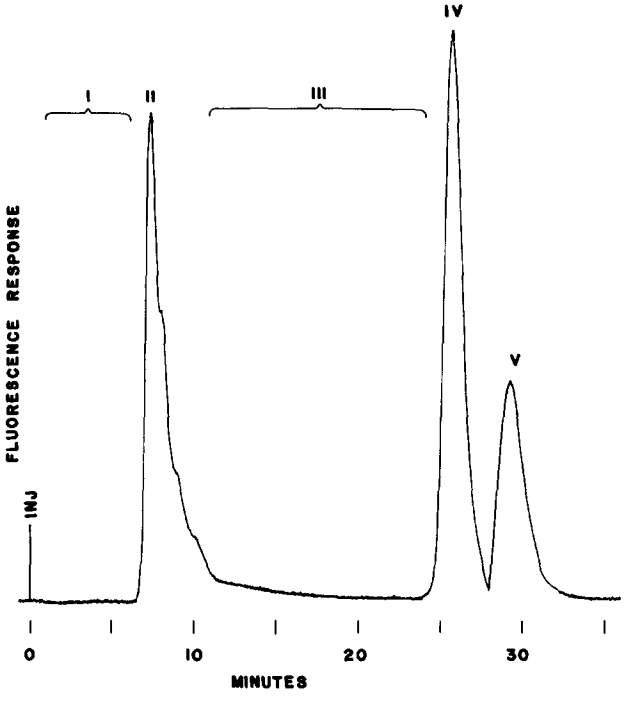

Figure 2
Chromatogram of clomiphene isomers as their post-column photoinduced phenanthrene analytes. The sample injected was obtained by extraction of spiked human plasma. I is the time for appearance of solvent front, which includes the reaction coil exposure time of 5.9 min; II represents co-extracted plasma contaminants; III represents time for elution of polar metabolites; IV is E(*trans*)-clomiphene; and V is Z(*cis*)-clomiphene.

(or volume), which was 5.9 min (or 8.9 ml), resulted in calculation of true chromatographic capacity factors, which were found to be $k' = 12.9$ and $k' = 10.6$ for the Z and E isomers, respectively (separation factor, $\alpha = 1.2$). The retention times of 21 min (E-isomer) and 24 min (Z-isomer) were a result of both choice and chance. The normal-phase system reported by Harman *et al.* [4], was characterized by late elution of polar metabolites. Therefore, in the present reversed-phase system it was desired to allow time for the elution of these polar metabolites between elution of the solvent front or plasma co-extracts and the intact clomiphene isomer peaks. It was found necessary to use the chromatographic conditions described above to maintain appropriate separation of the isomers; these conditions resulted in the retention characteristics demonstrated in Fig. 2.

Resolution of the isomers of clomiphene demonstrated a strong dependence on the concentration of triethylamine in the mobile phase. At higher levels of triethylamine both isomers could be eluted at much lower retention volumes; however, under these conditions there was little or no resolution of the isomers. In the absence of triethylamine the isomers were well resolved, but retention volumes were excessively large and peak shape was compromised. Inclusion of a small amount of amine modifier in the mobile phase to achieve separation resulted in good resolution of the Z and E isomers with suitable retention volumes and good peak shape.

Quantitation and extraction efficiency of clomiphene isomers

The individual isomers of clomiphene were determined by measurement of chromatographic peak areas. Linear calibration graphs of peak area against amount injected over various working ranges were routinely constructed and repeated. Variation in fluorescence response factors resulted in variations of less than a few percent in peak area in the calibration graphs on a day-to-day basis. Figure 3 shows a log–log plot of peak area against amount (in ng) of free base injected. This plot is a compilation of several calibration experiments based upon extractions of spiked human plasma (circles), extractions of standard aqueous solutions (squares), and direct injection of standard solutions (triangles). If the extraction procedure is performed as described above, the range (0.03–300 ng of injected amounts of drug corresponds to a plasma concentration range of 0.06–600 ng/ml.

The plotted abscissal values in Fig. 3 of the amount injected for the extracted spiked human plasma (circles) and the extracted standard aqueous solutions (squares) assume quantitative extraction of these solutions. The co-linearity displayed in Fig. 3 between the extracted samples (spiked plasma and standard solutions) and the direct standard injections indicates a high and reproducible extraction efficiency. It was found that for injections of more than 0.5 ng, which corresponds to plasma and standard solution samples of 1 ng/ml, the extraction efficiency was 98 ± 4% (RSD). For injections of less than 0.5 ng, the extraction efficiency was 85 ± 7% (RSD).

Post-column reaction coil

The detectability of clomiphene and related compounds has been shown to be improved by the photochemically induced conversion of these compounds to their corresponding phenanthrene ring systems. Although the parent stilbene compounds are not fluorescent, their photochemical reaction products are highly fluorescent, enabling detection of quantities much lower than would be possible by direct UV detection.

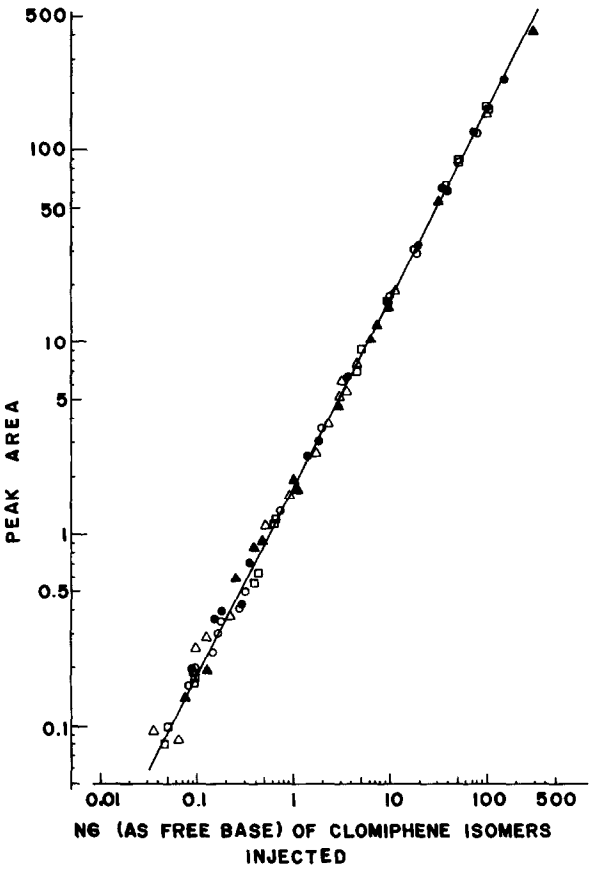

Figure 3
A log–log plot of peak area against ng of free base injected for both clomiphene isomers, demonstrating linearity of fluorescence response over four orders of magnitude. Solid symbols represent E(*trans*)-clomiphene; Open symbols represent Z(*cis*)-clomiphene; Circles represent extracted spiked human plasma samples; Squares represent extracted aqueous solution standards; and triangles represent direct injections of aqueous solution standards.

Methods on the analysis of tamoxifen utilize pre-column [6] and post-column conversion [9], whereas the normal-phase method for clomiphene utilizes post-column conversion of its isomers to their phenanthrene products [4]. Although both pre- and post-column conversion methods have been shown to give acceptable results, a post-column reaction was chosen for these studies. If photochemical reaction times are suitably short, use of a post-column reactor simplifies the analytical procedure. The reaction coil design described in the present work results in times of exposure of the compounds to the UV source such that conversions to the analyte are reproducible and give excellent sensitivity. Although photoinduced conversion to a single analyte was reported for tamoxifen [6], pre-column irradiation of a mixture of the clomiphene isomers results in formation of multiple fluorescent products. The chromatographic complexity resulting from an injection of the photochemical reaction mixture precluded a pre-column irradiation approach as an alternative analytical approach.

Although post-column reactors have been used extensively, they suffer from several problems, one of the most significant being band broadening in the reactor which can lead to serious losses in chromatographic resolution [10]. To minimize this band broadening several approaches have been made. One approach is segmenting the flowing stream in a coiled capillary reactor either with air or with an immiscible solvent. The second approach is deforming the tubing in a capillary reactor. Such deformation, accomplished by bending the tubing in alternate directions to create an oscillating pattern, serves to decrease band broadening by causing secondary flow patterns to be established within the flowing stream [11]. The dispersion seen when long lengths of coiled tubing are used arises from the laminar flow pattern established in the flowing stream. In wavy tubing, secondary flow patterns are established at low velocities in the moving stream and band broadening is substantially decreased.

The use of PTFE tubing to construct the reaction coil provides a flexible, UV transparent material with which to work [12]. Knitting this tubing provides a means of deforming the tubing into a wavy pattern and of keeping the tubing in a configuration maximal for UV irradiation [7, 8].

In order to obtain maximal conversion of clomiphene to fluorescent species, reaction coils of various lengths were constructed. The efficiency of conversion was determined by measuring peak area as a function of reaction coil length or reaction time. The final reaction coil of 18 was found to be optimal with respect to lamp geometry and reaction time. No reduction was found in detector response as reaction times were increased, unlike the results of Harman *et al.* [4]. This is no doubt due to the use of a lower intensity UV lamp in the present work.

The effect of the post-column irradiation coil on band broadening was investigated by determining the resolution of the Z and E isomers with no reaction coil, with a braided reaction coil and with an equivalent length of unbraided tubing. The UV absorbance detector was used in this series of experiments. For separation of isomers having retention times of 13.3 and 15.3 min with only the column in place, the effect of the braided coil was found to be substantial. Under these conditions the resolution (R_s) was found to be 1.14 with only the column, 0.89 with a 9-m braided reaction coil and 0.53 with 9-m straight PTFE tubing. The same experiment was repeated under different conditions where the retention times of the isomers were 17.5 and 20.6 min with only the analytical column. Under these conditions the resolution was found to be 1.07 with the column only, 1.00 with an 18-m braided reaction coil in place and 0.92 with 18 m straight PTFE tubing. It is evident that some loss in resolution occurs with the post-column reaction coil in line; however, it is also clear that deforming the tubing significantly decreases the band broadening brought about by the tubing. The less dramatic changes in resolution with larger retention volumes occur as the result of significant band broadening taking place during the residence time of the bands in the analytical column. It is expected that changes in resolution would become increasingly dramatic at shorter and shorter retention volumes.

Metabolite detection

The applicability of the method for determination of the intact (unmetabolized) isomers of clomiphene in plasma has been demonstrated in Figs 2 and 3. The applicability of the method to samples *in vivo*, both in respect of determination of the clomiphene isomers and detection of human metabolites, is shown in Figs 4 and 5. Figure 4 is a chromatogram derived from a blood sample drawn from a human volunteer prior to

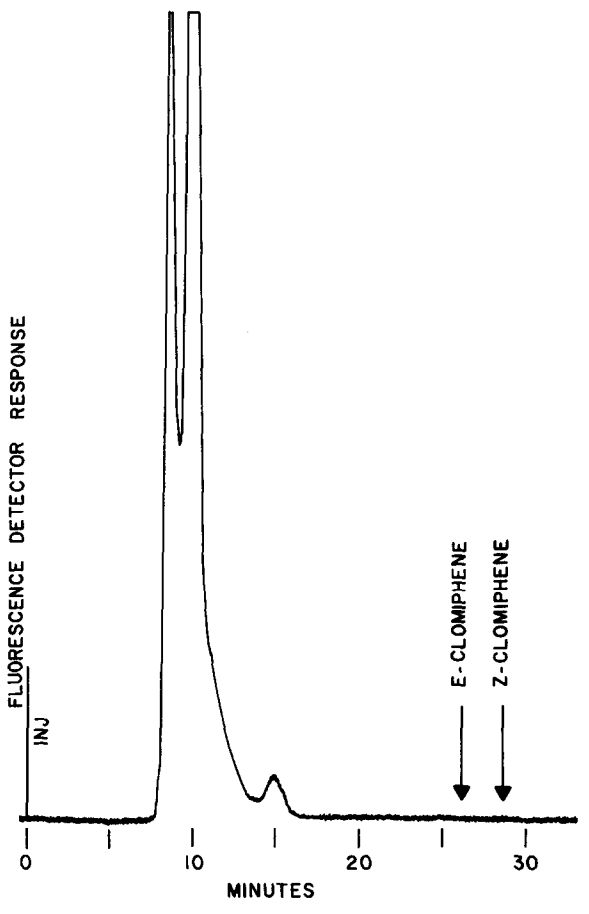

Figure 4
Chromatogram of a human plasma blank (before dosing) carried through the analytical procedure, showing co-extracted plasma contaminants. The arrows indicate the retention of the E and Z isomers of clomiphene.

clomiphene administration. Figure 5 is a chromatogram derived from a blood sample taken 6 h after administration of one 50-mg clomiphene citrate tablet to the same volunteer. The whole blood samples used in these experiments were drawn into vials containing heparin and immediately centrifuged. The resulting plasma was harvested and carried through the analytical sequence.

In Fig. 5, the peaks labelled III–VII are chromatographically resolved, but structurally uncharacterized, metabolites. Characterization of these metabolites will be the subject of a subsequent report. However, if the human metabolic pattern of clomiphene is similar to that of tamoxifen [5], it is probable that these metabolite peaks represent in part the N-de-ethylated and 4′-hydroxylated products of the isomers of clomiphene.

The peaks labelled VIII and IX in Fig. 5 are the E(*trans*) and Z(*cis*) isomers of clomiphene, respectively. The peak areas correspond to plasma levels 6 h after administration of 2.5 ng/ml of E clomiphene and 10.5 ng/ml of Z clomiphene. This ratio of E clomiphene/Z clomiphene is approximately 0.24, compared with the approximate ratio of 2.0 for the tablet dosage form. This ratio reversal for the isomers measured in

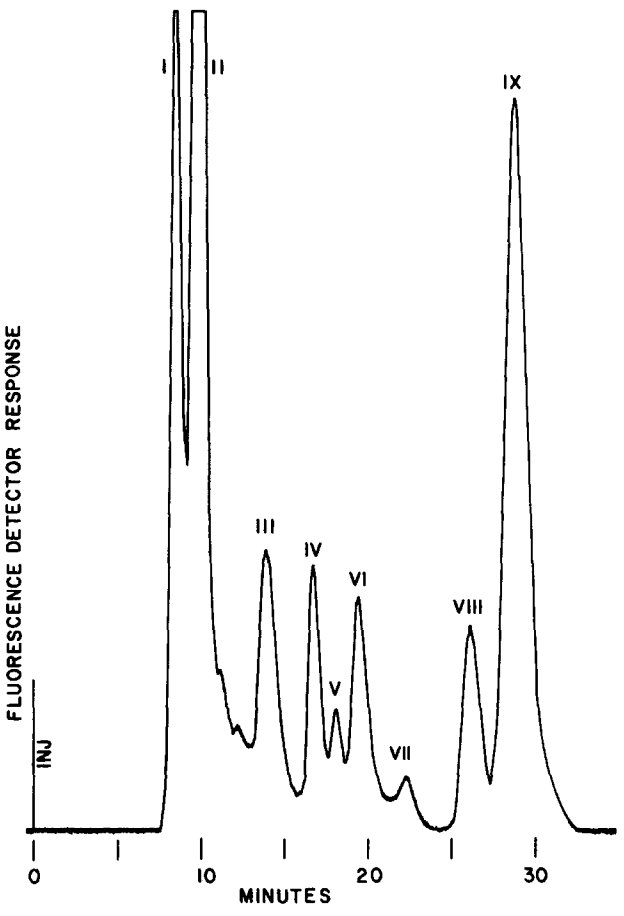

Figure 5
Chromatogram of a human plasma sample from blood drawn 6 h after the oral administration of a 50-mg clomiphene citrate tablet. I and II are co-extracted plasma contaminants; III–VII are structurally uncharacterized metabolites; and VIII and IX are the E(*trans*) and Z(*cis*) isomers of clomiphene, respectively.

plasma is an observation consistent with that previously noted by Harman *et al.* [4]. A subsequent report of a pharmacokinetic study conducted in 24 human volunteers will demonstrate that this ratio reversal is a result of a substantially more rapid elimination of the E isomer.

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