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Short Communication

Simple and rapid determination of clomiphene *cis* and *trans* isomers in human plasma by high-performance liquid chromatography using on-line post-column photochemical derivatization and fluorescence detection

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ABSTRACT

A validated, sensitive and rapid high-performance high-performance liquid chromatographic method has been developed for the analysis of both *cis* and *trans* isomers of clomiphene in human plasma. The method involves a new off-line pre-column solid-phase extraction for sample preparation of clomiphene from human plasma in the presence of an internal standard. Analysis was performed by isocratic elution on a LiChrospher 100 RP-18 5-μm column using on-line post-column photochemical derivatization, with fluorescence detection at 247 nm (excitation) and 378 nm (emission). The limit of quantitation was 0.75 ng/ml for the *cis* isomer and 1.25 ng/ml for the *trans* isomer. The precision and accuracy of method were between good laboratory practice (GLP) required limits.

INTRODUCTION

Clomiphene, 2-[4-(chloro-1,2-diphenylethenil)-phenoxy]-N,N-diethylethanamine, is a stilbene-based synthetic antioestrogen compound (Fig. 1) used to induce ovulation for anovulatory women and for the treatment of oligospermia in men. The oral dose of clomiphene citrate is usually 50 mg per day. Clomiphene shows good absorption after oral administration to humans. The biological half-life is five to seven days [1,2]. The *E* (*trans*) isomer of clomiphene is absorbed and eliminated more rapidly than the *Z* (*cis*) isomer [3]. Clomiphene and its metabolites have an enterohepatic recirculation, and the compounds are still present in low concentration in plasma up to three weeks after administration [3]. Clomiphene and its phenolic metabolites accumulate in oestrogen receptor-containing tissues, which suggests that they are the active metabolites of the drug [4]. The USP XXII specifies between 30 and 50% for the *Z* isomer in clomiphene citrate preparations [3]. The *Z* isomer has an antioestrogenic and the *E* isomer has an oestrogenic activity [5].

This paper describes a rapid and sensitive high-performance liquid chromatographic (HPLC) method for the quantification of clomiphene isomers in human plasma. The separation of *Z*

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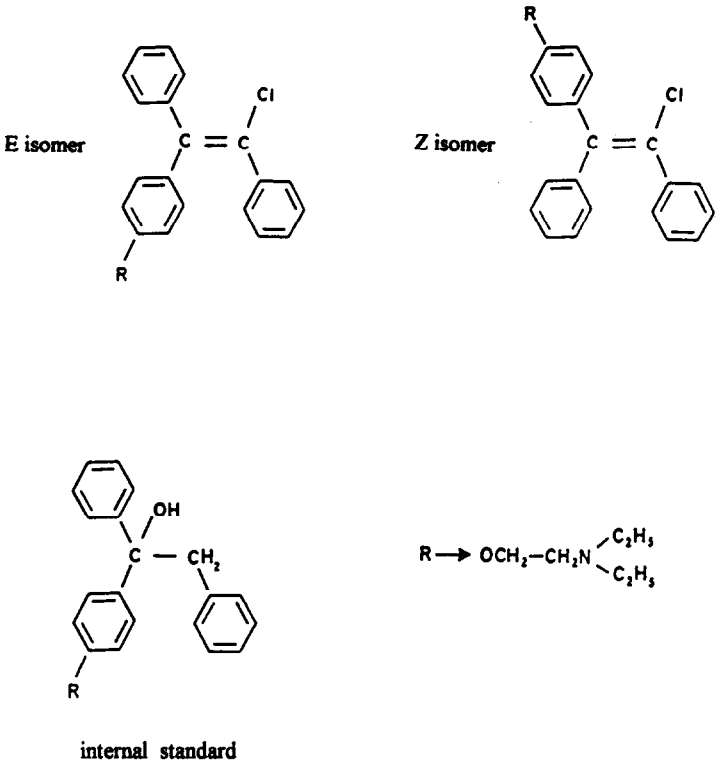

Fig. 1. Structures of clomiphene *Z* and *E* isomers and the internal standard.

and *E* isomers was achieved with a reversed-phase system. During derivatization (in an on-line Beam Boost photochemical unit) the stilbene base of isomers was converted by UV irradiation (254 nm) into intensively fluorescent phenanthrene compounds [6,7]. The HPLC method involves a new rapid and simple solid-phase extraction of clomiphene isomers from human plasma. Other advantages of the method are that it is more sensitive and faster than previously reported methods [7–9], and the standardized on-line post-column fluorescence derivatization in a photochemical reaction unit involving a knitted reaction coil.

The method was developed for a bioequivalence study and drug monitoring.

EXPERIMENTAL

Materials

Clomiphene citrate and 1,4-diethylaminoethoxy)phenyl-1,2-diphenylethanol (internal standard, Fig. 1) were manufactured by EGIS Pharmaceuticals (Budapest, Hungary). The applied clomiphene citrate contained 37.25% of the *Z* isomer and 67.75% of the *E* isomer. Clostilbegyt, 50-mg tablets, were supplied from EGIS Pharmaceuticals.

Acetonitrile and methanol (HPLC grade) were obtained from Fluka (Buchs, Switzerland), water (chromatographic grade) and sodium chloride (analytical-reagent grade) from Merck (Darmstadt, Germany) and ammonium chloride and potassium carbonate (analytical-reagent grade) from Reanal (Budapest, Hungary).

Instrumentation

The analytical column was a LiChrospher 100 RP-18 (5 μm, 250 mm × 4 mm I.D.) from Hewlett-Packard (Palo Alto, CA, USA). A Hewlett-Packard HP 1090 Series II/M liquid chromatograph and an HP 1046A programmable fluorescence detector were used. The control of

the complete system and the evaluation of chromatograms were carried out from an HPLC ChemStation (Pascal Series HP-79994A HPLC operating software), using an HP 9000 Series 300 computer (Hewlett-Packard). The Beam Boost photochemical reaction unit (ICT Chemik, Wien, Austria) was incorporated on-line between the chromatograph and the detector.

A vacuum station for solid-phase extraction (SPE) (Supelco, Gland, Switzerland), equipped with Bond Elut C_{18} (1 ml, 40 μm particle diameter) SPE extraction cartridges (Analytichem International, Harbor City, CA, USA) was used. Durapore membrane filters (0.45 μm pore diameter) (Millipore, Bedford, MA, USA) were used to filter the mobile phase.

Chromatographic conditions

The separation of the *Z* and *E* isomers and the internal standard was achieved on LiChrospher 100 RP-18 phase. The eluent consisted of 950 ml of acetonitrile, 30 ml of methanol, 20 ml of water, 4 ml of 1% ammonium chloride, and 8 ml of 1% potassium carbonate; it was filtered through a membrane filter and purged with helium to expel dissolved oxygen. The flow-rate was 1.0 ml/min at 70 bar pressure. The column was thermostatted at 30°C. The sample injection volume was 20 μl.

The post-column photochemical derivatization of the clomiphene isomers and the internal standard was accomplished on-line in the 15 m × 0.3 mm I.D. reaction coil (PTFE tube) knitted on the mercury lamp (UV wavelength 254 nm) of the Beam Boost photochemical reaction unit.

The excitation and emission wavelengths of the fluorescence detector were 247 and 378 nm, respectively. The detector lamp flash frequency was 55 Hz in the higher concentration range and 220 Hz in the lower.

Standard solutions

Spiked plasma samples were prepared from collected human plasma that was stored at −20°C. Stock solutions of clomiphene citrate and the internal standard were prepared in methanol to 1 mg/ml concentration, and stored at −20°C for one week.

A calibration curve was obtained using spiked human plasma samples containing clomiphene citrate (37.5% *Z* isomer and 67.75% *E* isomer) in the following concentrations: 2.0, 5.0, 10.0, 25.0, 50.0, 100.0, 500.0, 1000.0, 2000.0 ng/ml; the internal standard concentration was 500 ng/ml.

Sample preparation

The Bond Elut C_{18} SPE columns (1 ml) were activated with 1 ml of methanol and 1 ml of water using the vacuum station. A 0.5-ml volume of 3 *M* NaCl and 1 ml of water were added to 1 ml of spiked plasma; the mixture was transferred to Bond Elut microcolumns. The sample was washed with 3 ml of water, and the compounds of interest were eluted with 3 ml of methanol, which was evaporated to dryness under a stream of nitrogen in a water-bath at 50°C. The residue was redissolved in 1 ml of eluent, and 20 μl of resultant solution were injected via the autosampler.

RESULTS

Typical chromatograms of *Z* and *E* isomers and the internal standard are shown in Fig. 2. The retention times were 7.2 min for the *Z* isomer, 6.8 min for the *E* isomer and 4.7 min for internal standard. The resolution between the *Z* and *E* isomers was $R_s = 1.5$. The chromatogram of blank plasma did not show co-eluting disturbing peaks (Fig. 2C). A chromatogram of a plasma extract taken 2 h after administration of 50 mg of clomiphene citrate to a healthy volunteer is shown in Fig. 2D.

The calibration curves showed good linearity in the range 2–2000 ng/ml of clomiphene citrate. The correlation was $r = 0.9983$ in the lower concentration range (0.75–74.5 ng/ml) and $r = 0.9949$ in the higher concentration range (74.5–745 ng/ml) for the *Z* isomer and $r = 0.9978$ in the lower concentration range (1.25–62.5 ng/ml) and $r = 0.9987$ in the higher range (62.5–1255 ng/ml) of *E* isomer.

The limits of quantitation were 0.75 ng/ml for the *Z* isomer and 1.25 ng/ml for the *E* isomer in human plasma. The limit of detection was 0.4 ng/ml for both the *cis* and *trans* isomers of clomiphene.

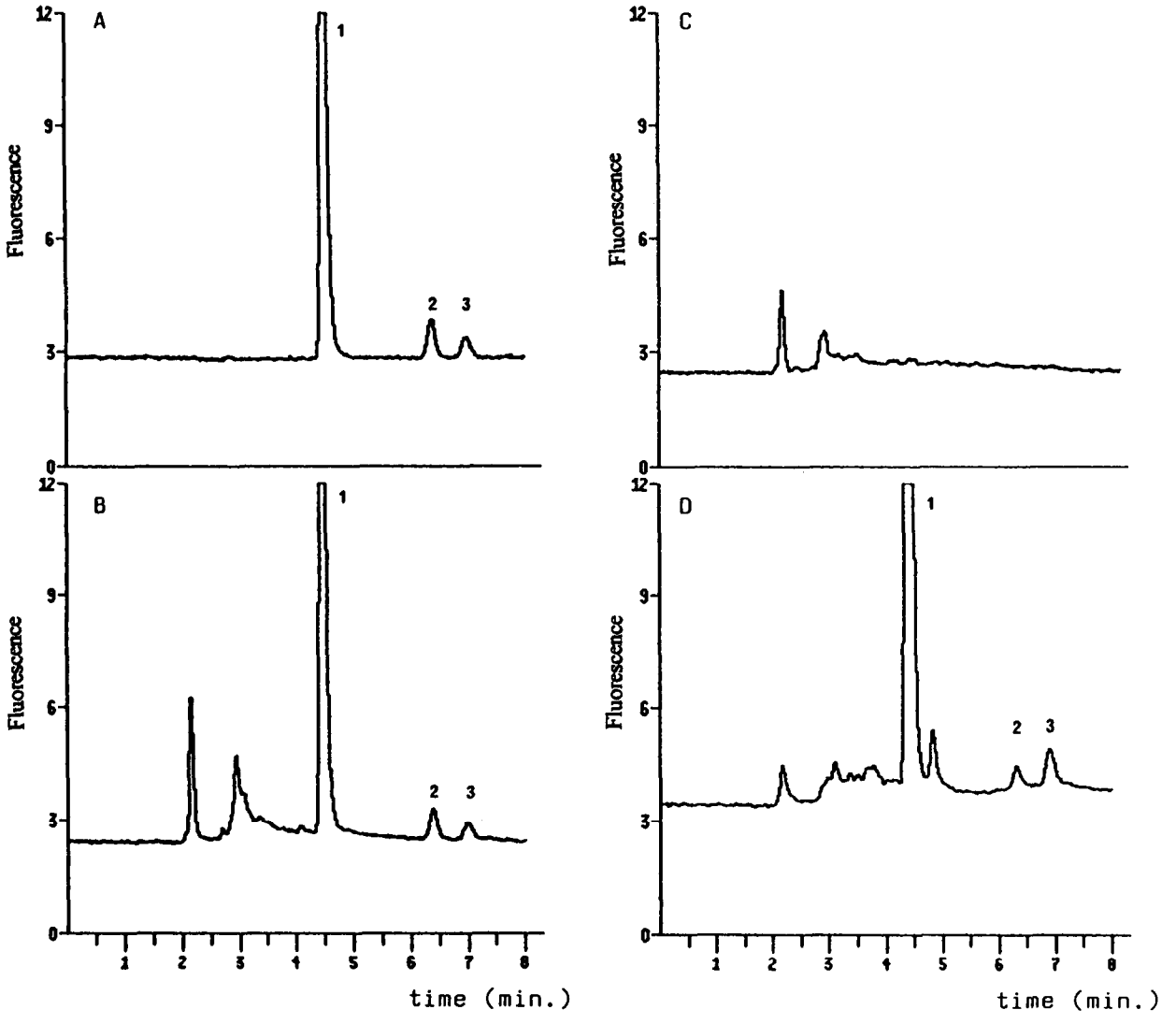

Fig. 2. Chromatograms of (A) a standard clomiphene (50 ng/ml) and internal standard (500 ng/ml) solution, (B) extracted spiked human plasma (50 ng/ml clomiphene and 500 ng/ml internal standard), (C) an extracted human blank plasma, and (D) healthy volunteer's plasma extract, 2 h after ingestion of Clostilbegyt (50-mg tablet). Peaks: 1 = internal standard; 2 = *trans* isomer; 3 = *cis* isomer.

The within-day precision (relative standard deviations, R.S.D.) and accuracy (%) were between 1.8 and 5.0% and −3.4 and 5.4% for the *cis* isomer; the equivalent values for the *trans* isomer were 2.6 and 4.7% and 0.5 and 4.9%. The day-to-day precision (R.S.D.) and accuracy (%) were between 4.9 and 18.9% and −6.6 and 8.2% for the *cis* isomer; the equivalent values for the *trans* isomer were 3.5 and 9.4% and −6.7 and 16.6%.

The precision of the chromatographic system (system suitability) was 2.0 and 3.6% (R.S.D.) at 9.31 and 37.25 ng/ml for the *Z* isomer, and 1.5 and 1.4% (R.S.D.) at 15.69 and 62.75 ng/ml for the *E* isomer.

The 50, 100 and 200 ng/ml spiked concentrations of clomiphene were stable in human plasma after storage for a year at −20°C.

The recoveries were 75 ± 5% for the *Z* isomer,

$62 \pm 6\%$ for the *E* isomer and $94 \pm 3\%$ for the internal standard.

CONCLUSION

This new validated method, involving SPE for sample preparation and HPLC separation with on-line post-column photochemical derivatization and fluorescence detection, can be used to determine clomiphene isomers in low (0.75 ng/ml *Z* and 1.25 ng/ml *E* isomer) concentrations in human plasma. It is suitable for the investigation of pharmacokinetic properties and for bioequivalence studies and drug monitoring.

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