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Journal of Chromatography B, 847 (2007) 296–299
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

# Determination by liquid chromatography–mass spectrometry of clomiphene isomers in the plasma of patients undergoing treatment for the induction of ovulation

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Received 3 August 2006; accepted 8 October 2006
Available online 7 November 2006

**Abstract**

A rapid, sensitive and selective LC–MS method is described for the simultaneous determination of zuclomiphene and enclomiphene in plasma from patients undergoing treatment with clomiphene citrate for the induction of ovulation. Samples spiked with *N*-didesmethyltamoxifen, the internal standard, were extracted into methyl tertiary butyl ether. The compounds were separated on a Luna $C_{18}$ analytical column, and a mobile phase of methanol–water (70:30 v/v) containing 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. The limits of determination were 35 pg/ml and 7 pg/ml for zu- and enclomiphene, respectively. Within-day coefficients of variation ranged from 2.1% to 7.2%.


*Keywords:* Clomiphene citrate; Zuclomiphene; Enclomiphene

## 1. Introduction

Clomiphene citrate (CC) is a synthetic anti-oestrogen, structurally related to diethylstilbestrol, and is a first choice therapy to treat women with absent or irregular ovulation due to hypothalamic-pituitary dysfunction associated with normal basal concentrations of endogenous oestradiol [1,2]. CC is administered as a mixture of its two geometric isomers, enclomiphene (E) and zuclomiphene (Z) citrate (Fig. 1) in the ratio 62%:38% [3].

Recent reports have indicated that a number of individual patient characteristics are responsible for the success or failure of treatment with CC [1,4–7]. However, a factor affecting response that has not been considered is that of plasma clomiphene concentration as a measure of drug exposure [1]. Individualization of CC dose based on the concentration of its isomers in plasma is currently the subject of study in our Unit. For this work, a selective analytical technique for the determination of clomiphene isomers in human plasma was required. Previous methods for the measurement of the isomers have relied on HPLC using post-column photochemical derivatisation followed by fluorescence detection [8–11]. Although these methods reported detection limits of less than 1 ng/ml, some required the use of up to 5 ml of plasma per sample. In order to carry out pharmacokinetic studies in humans, a more sensitive method capable for the measurement of low concentrations of clomiphene isomers in plasma was desirable. The present paper describes a highly sensitive and selective method using liquid chromatography–mass spectrometry (LC–MS).

## 2. Experimental

### 2.1. Chemicals

Zu- and enclomiphene citrate were gifts from Aventis Pharma (Frankfurt, Germany). *N*-Didesmethyltamoxifen was obtained from Klinge Pharma (Munich, Germany). All other chemicals were of analytical grade and used without further purification.

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doi:10.1016/j.jchromb.2006.10.019

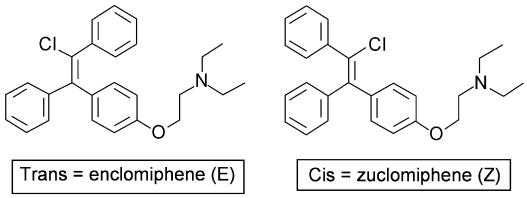


Fig. 1. The two geometric isomers of clomiphene.

### 2.2. LC–MS instrumentation and conditions

Analysis was performed on a Waters™ 2690 separation module (Waters, Watford, UK) coupled to a platform LC single quadruple mass spectrometer (Micromass, Altrincham, U.K), equipped with an atmospheric pressure ionisation (APCI) source and a crossflow counter electrode. An APCI pin voltage of 4.2 kV and a cone voltage of 17 V were used. The source heater was set at 150 °C and the APCI heater at 500 °C. The drying nitrogen flow rate was 402 l/h.

Chromatographic separation was performed on a Luna $C_{18}$ analytical column (3 μm particle size, 100 mm × 4.6 mm I.D.) coupled to a guard $C_{18}$ precolumn (Phenomenex, Cheshire, UK). A mobile phase of methanol–water containing 0.05% trifluoracetic acid (70:30v/v) was delivered isocratically at 1 ml/min.

### 2.3. Sample preparation

*N*-didesmethyl tamoxifen (5 ng) (internal standard), and 5 ml of methyl tertiary butyl ether were added to glass tubes containing 1 ml plasma. The samples were mixed for 2 min on a Dade® multi tube vortexer (Alpha Laboratories Ltd., Hampshire, UK.). After centrifugation the upper organic phase was separated and evaporated to dryness under nitrogen using a Techne® sample concentrator (Jencons Scientific Ltd., Bedfordshire, UK.). The sample residue was reconstituted in 150 μl of mobile phase. Because the cloudy appearance of the sample prevented its direct injection onto the LC–MS, it was centrifuged at 13,000 rpm for 5 min in a MSE microfuge (Sanyo Gallenkamp plc, Loughborough, UK.), and 100 μl of the resulting supernatant was injected.

### 2.4. Calibration curves

Blank plasma samples were spiked with known quantities of zu- (0–31.2 ng/ml) and (0–6.8 ng/ml) enclomiphene and internal standard (5 ng). Calibration curves were constructed by plotting the peak area ratios of drug to internal standard against drug concentration. The lines of best fit were determined using least square linear regression.

### 2.5. Assay precision

The precision of the assay was defined by the co-efficient of variation (CV), which is the standard deviation expressed as a percentage of the mean of a series of replicates. Within-day precision was determined by preparing and analysing replicates on the same day. Between-day precision was determined by preparing and analysing replicates on different days over a period of five weeks.

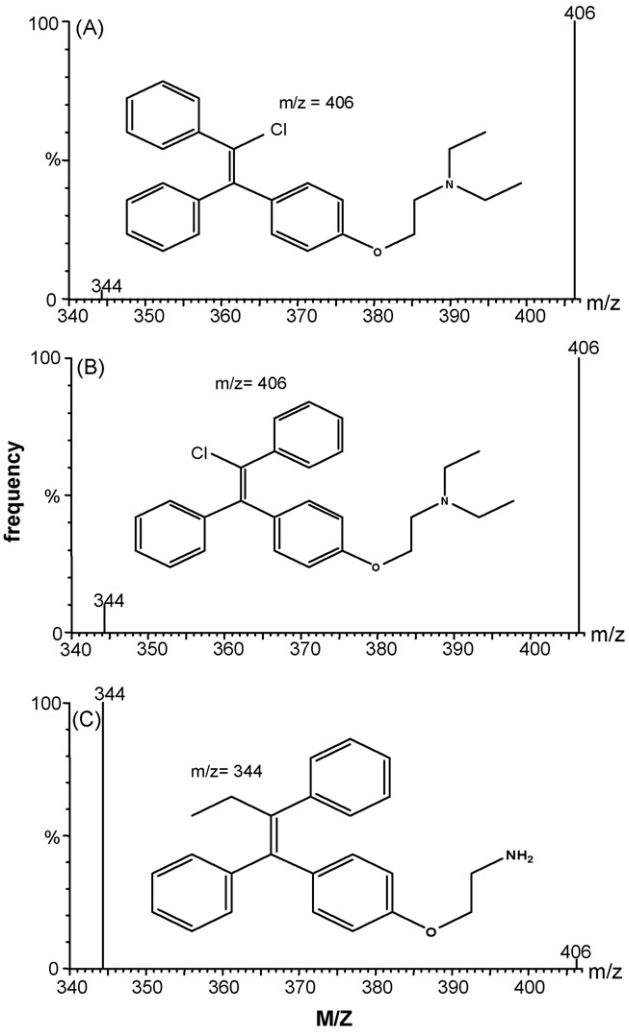


Fig. 2. Mass spectra of: (A) zuclomiphene; (B) enclomiphene; and (C) *N*-didesmethyltamoxifen (internal standard), obtained from an extracted sample containing all three compounds.

## 3. Results

Mass spectra of the clomiphene isomers and *N*-didesmethyltamoxifen are shown in Fig. 2. Single Ion Monitoring was used and the ions at *m/z* of 406.3 and 344.3 were selected for subsequent analysis. Under the chromatographic conditions used, zuclomiphene (retention time 3.35 min), enclomiphene (retention time 4.04 min) and *N*-didesmethyltamoxifen (retention time = 5.66 min) gave rapidly eluting, fully resolved and essentially symmetrical peaks (Fig. 3).

Calibration curves for both isomers were linear (Fig. 4). Assay precision data are shown in Tables 1 and 2. The limit of determination, defined as the lowest concentration yielding a signal to noise ratio higher than 3, was 35 pg/ml and 7 pg/ml for zu- and enclomiphene, respectively.

The chromatogram resulting from the analysis of a plasma sample obtained from an anovulatory patient 2 h after a second daily dose of 50 mg CC, is shown in Fig. 3.

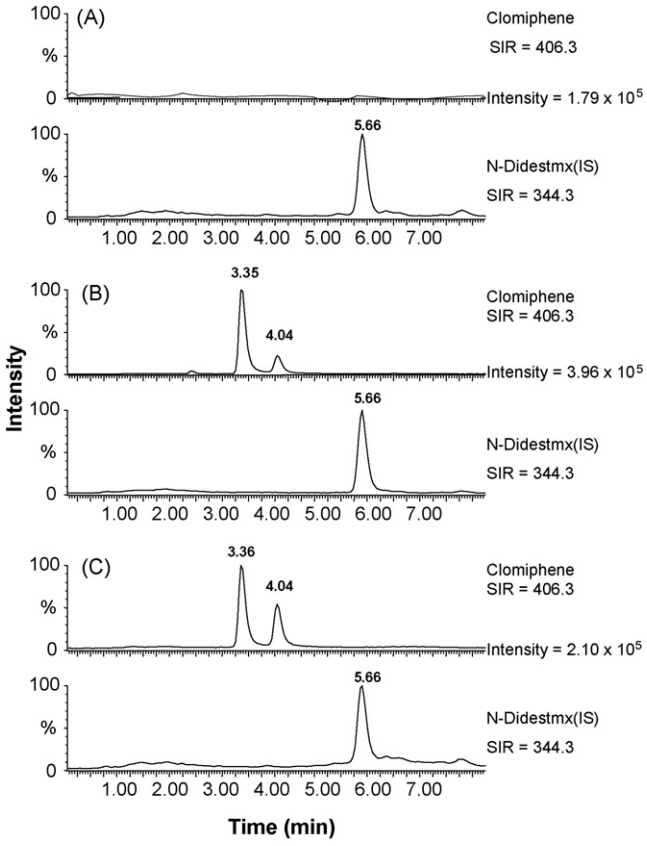


Fig. 3. Reconstructed mass chromatograms produced from the analysis of: (A) blank plasma spiked with internal standard; (B) blank plasma spiked with 21.7 ng/ml and 4.1 ng/ml of zu- (retention time = 3.35 min) and enclomiphene (retention time = 4.04 min), respectively; and (C) a patient sample containing 6.9 ng/ml zu- and 3.6 ng/ml enclomiphene, obtained two hours after a second daily dose of 50 mg CC.

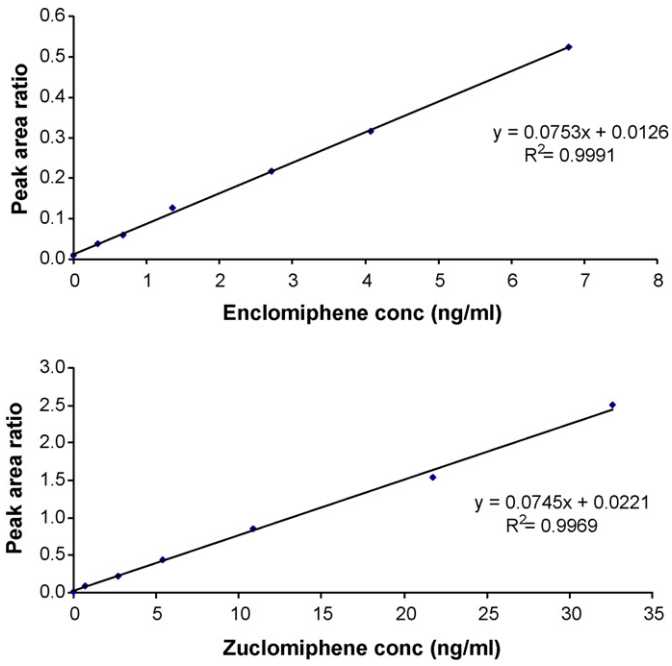


Fig. 4. Representative calibration curves for zu- and enclomiphene.

## 4. Discussion

HPLC with post column UV derivatisation and fluorescence detection has been previously used to measure clomiphene isomers in patients [8–11]. Using a modification of these methods with 2 ml of plasma, our laboratory obtained reproducible standard curves, intra-assay CVs of less than 10% at 6 ng/ml and limits of determination of 0.5 ng/ml and 0.25 ng/ml, respectively, for zu- and enclomiphene, However, a major difficulty encountered was the presence of endogenous interfering peaks in some blank plasma and patient samples. Attempts to resolve these peaks from those associated with clomiphene isomers by manipulation of mobile phase pH, triethylamine content and solvent composition were unsatisfactory and often resulted in prohibitively long retention times. Re-analysis using the present LC–MS method of samples previously measured using UV derivatisation and fluorescence detection, resulted in the ability

Table 1
Intra-assay coefficients of variation for clomiphene isomers

| Zuclomiphene concentration (ng/ml) | Co-efficient of variation (%) | Number of replicates | Enclomiphene concentration (ng/ml) | Co-efficient of variation (%) | Number of replicates |
|---|---|---|---|---|---|
| 0.68 | 2.1 | 6 | 0.68 | 5.5 | 6 |
| 5.43 | 3.5 | 6 | 2.5 | 4.5 | 5 |
| 10 | 3.2 | 5 | 4.07 | 4.6 | 6 |
| 21.7 | 7.2 | 6 | 5.43 | 2.4 | 6 |
| 40 | 5.1 | 6 | 10 | 6.8 | 6 |

Table 2
Inter-assay coefficients of variation for clomiphene isomers

| Zuclomiphene concentration (ng/ml) | Coefficient of variation (%) | Number of replicates | Enclomiphene concentration (ng/ml) | Coefficient of variation (%) | Number of replicates |
|---|---|---|---|---|---|
| 0.68 | 20.9 | 5 | 0.68 | 20.1 | 5 |
| 5.43 | 15.7 | 5 | 2.72 | 15.9 | 4 |
| 21.72 | 9.8 | 5 | 4.07 | 14.3 | 5 |
| 32.58 | 12.8 | 4 | 6.79 | 13.9 | 4 |

to measure concentrations of enclomiphene that were previously undetectable, Furthermore, zuclomiphene concentrations measured by the HPLC/UV/fluorescence method had been overestimated in some cases.

In summary, we describe a rapid, sensitive and most importantly selective method for the analysis of clomiphene isomers in plasma. Its high sensitivity enables lower plasma volumes to be used than previously published techniques. The method was found to be readily applicable to the analysis of plasma samples from patients undergoing CC therapy over a range of doses.

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