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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CLOMIPHENE USING POST-COLUMN ON-LINE PHOTOLYSIS AND FLUORESCENCE DETECTION

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## SUMMARY

A method has been developed for the extraction and quantitation of the ovulatory stimulant drug clomiphene from blood plasma. The *cis*- and *trans*-isomers were separated by normal-phase chromatography using chloroform—methanol as the mobile phase. After eluting from the column, the clomiphene was passed through a PTFE photolysis coil irradiated by a powerful UV lamp, resulting in conversion of the isomers to highly fluorescent species. The derivatised material was then detected using a fluorescence spectrometer. Use of this method enables a substantial improvement in sensitivity over UV detection and has permitted the measurement of plasma clomiphene levels in patients receiving clomiphene therapy.

## INTRODUCTION

Clomiphene (Clomid, Merrell, Fig. 1) is a non steroidal triphenylethylene compound which is currently used as an ovulatory stimulant. It is the drug of choice in the normoprolactinaemic anovulatory woman who has gonadotrophin and oestrogen production. In properly selected patients 70% can be expected to ovulate, however the risk of multiple pregnancy in those who conceive is about 20% [1].

The mode of action of the drug is not clearly understood but it appears that clomiphene stimulates the release of gonadotrophin by a direct action

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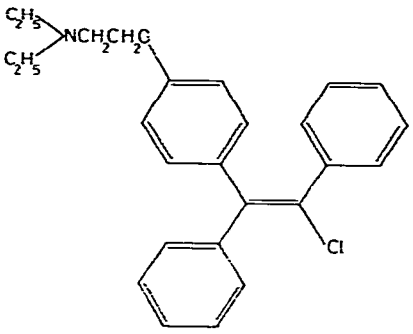


Fig. 1. Structure of clomiphene.

on the hypothalamic/pituitary axis and by reducing the inhibitory influence of endogenous oestrogens.

Studies with $^{14}C$-labelled clomiphene [2, 3] indicate that it is readily absorbed after oral administration and is excreted mainly via the faeces. The half-life for the administered radioactivity in an oral tracer dose is five days. However, the drug is still present in the faeces up to six weeks following administration, and thus enterohepatic recycling is suspected [4]. No information is available on the plasma levels present after a single dose but studies of a closely related drug, tamoxifen, have indicated concentrations of 80 pmol/ml after a single dose of 10 mg/m$^2$ body surface area [5]. After administration of a single dose of 100 mg of clomiphene citrate, peak concentrations of 25—250 pmol/ml of plasma could therefore be expected. Thus an analytical method capable of detecting clomiphene plasma levels below 1 ng/ml is required.

Clomiphene as normally administered consists of a mixture of the *cis*- and *trans*-isomers. In order to derive meaningful pharmacokinetic data from such samples, the method utilised to determine the drug should be able to distinguish between the two isomers, as studies have shown that only the *cis*-isomer is active [6, 7]. Clomiphene however has a relatively low extinction coefficient for UV absorption ($\epsilon$ = 10,450 l/mol cm at 298 nm) which would not permit quantitation of nanogram amounts of the drugs using UV detection.

Recent work on tamoxifen [5, 8—10] has made use of the conversion of stilbene-based molecules by UV irradiation to highly fluorescent phenanthrene derivatives. This conversion has also been used in the assay of diethylstilbestrol [11].

In this paper we describe a method to analyse clomiphene levels in plasma. The isomers are extracted from the plasma and separated by high-performance liquid chromatography (HPLC) on a silica column. The eluted isomers are passed through a photolysis coil irradiated by a powerful UV lamp, resulting in conversion of the isomers to highly fluorescent species. These derivatised products are then detected using a fluorescence spectrophotometer. The use of photochemical reactions for post column derivatization has recently been reviewed [12].

## EXPERIMENTAL

### *Reagents*

Analytical-grade chloroform, methanol and diethyl ether were redistilled before use. It was necessary to ensure that the chloroform and methanol used in the HPLC mobile phase were very carefully dried to maintain constant retention times. Thus methanol was redistilled from magnesium and iodine and stored over molecular sieve while chloroform was redistilled from anhydrous calcium chloride and stored over molecular sieve. Diethyl ether was redistilled and stored over sodium. *trans*-Clomiphene citrate and clomiphene citrate (a mixture of the *cis*- and *trans*-isomers) were generously donated by Wm.S. Merrell Company (Sydney, Australia).

### *Equipment*

The HPLC system (Fig. 2) incorporated a Perkin-Elmer Series 3B pump fitted with a septum injector, and either a Perkin-Elmer Model 3000 or a Model 650-10S fluorescence spectrometer. For some experiments a Perkin-Elmer Model LC75 UV detector was used. Separation was achieved on a DuPont Zorbax (6 $\mu$m) Sil silica column (25 cm × 4.6 mm I.D.). The mobile phase was chloroform—methanol (80:20). The photochemical reactor consisted of a 3-m length of 0.3 mm internal diameter PTFE-tubing wound around the silica window section of the cooling waterjacket on an Hanovia medium-pressure photochemical mercury lamp. The exposed sections of the lamp were wrapped in aluminium foil to reduce stray radiation.

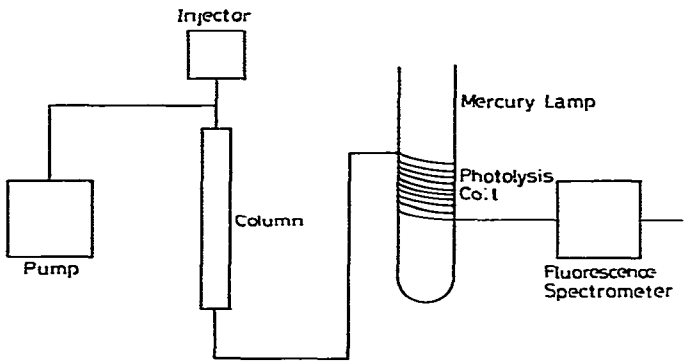


Fig. 2. HPLC system for on-line photolysis.

### *Procedure*

Samples of whole blood, taken by venipuncture at varying times after administration of the dose, were obtained from patients undergoing clomiphene therapy. The blood was collected in tubes containing lithium heparin, which were then centrifuged. The plasma was transferred to small sample vials and stored at −20°C until analysed.

Samples of plasma (3 ml) were transferred into 20-ml centrifuge tubes containing 1 ml of borate buffer, pH 9. The tube was vortexed and 9 ml of redistilled AR diethyl ether were added. The tube was vortexed for a further

2 min to ensure thorough mixing and then centrifuged at 1600 *g* for 15 min. The ether layer was transferred to a 10-ml centrifuge tube and dried by the addition of 1 g of anhydrous sodium sulphate. The tube was then centrifuged at 1600 *g* for 10 min and the ether layer transferred to another 10-ml centrifuge tube. The ether was evaporated to dryness with a stream of air and the extracted material was redissolved in 75 μl of chloroform—methanol (80:20). The tube was vortexed for 2 min to dissolve any material adhering to the walls of the centrifuge tube, and 10 μl of the resultant solution were injected into the chromatograph. The extraction efficiency was 70% at 30 ng/ml.

## RESULTS AND DISCUSSION

### *Chromatography*

A representative chromatogram obtained from a standard solution of clomiphene (1 μg/ml) in methanol is shown in Fig. 3. Separation of the *cis*- and *trans*-isomers can be clearly seen and the areas of the peaks are in proportion to the relative concentrations of the respective isomers in the solution (*cis* 55%, *trans* 45%). By comparison with a chromatogram obtained using a UV detector set at 298 nm inserted before the photolysis coil, there is a significant improvement in signal-to-noise ratio with little degradation of the chromatographic resolution.

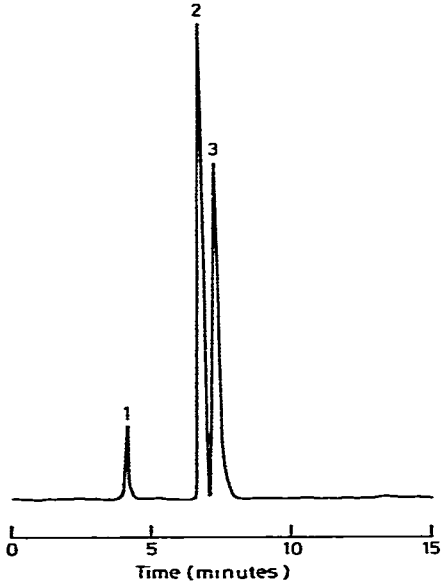


Fig. 3. HPLC chromatogram of a standard solution of clomiphene in methanol (1 μg/ml). Peaks: (1) impurity peak from methanol, (2) *cis*-clomiphene and (3) *trans*-clomiphene.

If the mercury lamp is switched off before the elution of the clomiphene isomers from the column, no signal is obtained from the fluorescence spectrometer.

Emission and excitation wavelengths of the photolysed derivatives were obtained by dissolving clomiphene in the mobile phase and exposing 3-ml

quartz cuvettes containing the solution to a UV lamp for 30 min. The emission and excitation spectra were scanned, and the solutions, which were previously not fluorescent, emitted strongly at 367 nm when excited at 257 nm.

Due to the on-line nature of the derivatisation, lengthy residence times of the eluted isomers within the photolysis coil would result in photochemical destruction of the derivatised isomers while short transit times would result in incomplete conversion of the clomiphene isomers. The variation in photolysis yield with flow-rate was determined by injecting a standard solution of clomiphene at varying flow-rates. The flow-rate response of the detector itself was measured by substituting naphthalene injections for the clomiphene and the photolysis flow-rate response was then corrected to yield the optimum flow-rate of 0.8 ml/min for the 3-m coil.

Linear calibration graphs passing through the origin were obtained for both isomers over a concentration range of 0—45 ng/ml after extraction from spiked plasma samples and were constructed from a measurement of the peak heights of the derivatised isomer peaks. Correlation coefficients of 0.989 and 0.984 were obtained for the *cis*- and *trans*-isomers respectively. An internal standard was not used in the assay since, despite an extensive evaluation, a suitable compound has not yet been found which satisfies the numerous criteria for an adequate internal standard.

Minimum detectable levels (signal-to-noise ratio $> 2$) for the two isomers were determined to be 145 fmol (60 pg) using a Perkin-Elmer 650-10S fluorescence spectrometer. This represents a minimum detectable level of 350 pg/ml in plasma. On the Perkin-Elmer Model 3000 fluorescence spectrometer the minimum detectable level for each isomer was 615 fmol (250 pg), whilst with UV absorption detection of the underivatised isomers the minimum detectable level was 12.5 pmol (5 ng).

*Fluorescence spectroscopy*

Stop-flow fluorescence spectra were obtained from plasma extracts by halting the flow of the mobile phase during elution of the peak, and scanning the emission and excitation spectra. The results for the *cis*-isomer are shown in Fig. 4 and can be seen to correlate very well with the stop-flow spectra standards. The spectra obtained from the *trans*-isomer were similar to those shown in Fig. 4. The spectra have been corrected for the slight fluorescence observed from the chloroform—methanol solvent when excited at 257 nm.

The structures of the photolysis products have not yet been confirmed but by analogy with the work on tamoxifen [5, 10] it is expected that the reaction will involve ring closure to form the phenanthrene derivatives. This conclusion is also in agreement with the conversion of stilbene and triphenylethylene to phenanthrene derivatives by UV irradiation [13, 14].

Both the *cis*- and *trans*-isomers will form phenanthrenes of very similar structure and would therefore be expected to show similar emission and excitation spectra, as is observed.

*Blood levels*

The analytical procedure described above has been used in a preliminary study of the plasma clomiphene levels of a group of patients undergoing clomiphene therapy.

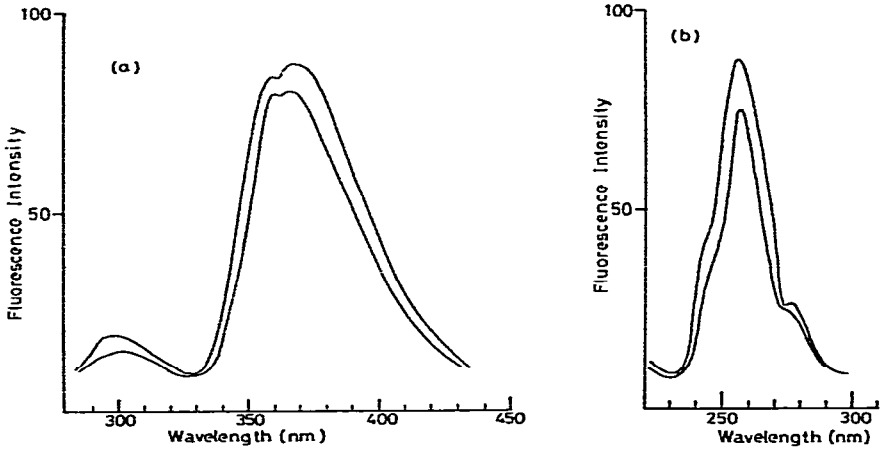


Fig. 4. Fluorescence spectra obtained by halting the flow of mobile phase during elution of *cis*-clomiphene. (a) Emission spectra with excitation at 257 nm; (b) excitation spectra with emission monitored at 367 nm. The upper traces are from spiked plasma samples while the lower are from a patient receiving clomiphene therapy.

A sample chromatogram is shown in Fig. 5 and the levels tabulated in Table I. The results show several interesting features. First, although the dosage form as administered contains 55% of the *cis*-isomer, the concentrations of the two isomers extracted from the plasma are not in the same ratio. The concentration of the *cis*-isomer has apparently fallen to approximately 35% of the total isomer concentration. This may be due to a number of factors relating to the distribution and metabolism of the drug.

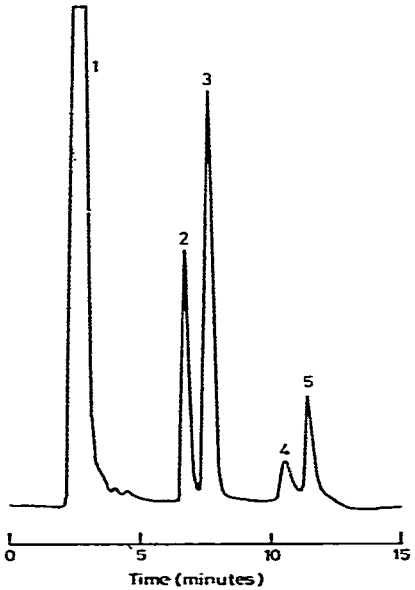


Fig. 5. HPLC chromatogram of a plasma sample from patient J. Peaks: (1) solvent peak, (2) *cis*-clomiphene, (3) *trans*-clomiphene, (4) and (5) clomiphene metabolites.

TABLE I

CLOMIPHENE LEVELS IN PATIENT PLASMA SAMPLES DETERMINED USING ON-LINE POST-COLUMN PHOTOLYSIS AND FLUORESCENCE DETECTION

| Sample identification | Time after dose (h) | Dosage (mg) | *Cis*-isomer (ng/ml) | *Trans*-isomer (ng/ml) |
|---|---|---|---|---|
| M | 2.0 | 100 | 6.9 | 23.1 |
| McG | 3.0 | 100 | 14.6 | 30.4 |
| A | 3.25 | 100 | 9.4 | 23.1 |
| S | 2.5 | 150 | 24.1 | 42.7 |
| J | 3.25 | 150 | 42.3 | 80.9 |
| B | 3.25 | 150 | 39.1 | >60 |

Secondly, from Fig. 5 the appearance of several other peaks can be observed. These must have a similar structure to clomiphene in order to be detected using the photolysis—fluorescence system, and it is postulated that these represent metabolites of the clomiphene. Another pair of peaks, not shown in Fig. 5, but of very much lower concentration is also observed.

Table I shows that there is good correlation between the dose administered and the levels observed in the plasma samples. The levels show a significant increase as the dosage level is raised and are around the expected values based upon the work on tamoxifen [5]. In all cases the *cis*:*trans* ratio is significantly below the ratio present in the dosage form.

Work is proceeding to confirm the identity of the derivatives, to elucidate the structure of the metabolites, and to investigate aspects of the pharmacokinetics of clomiphene. The results of these investigations will be presented in a subsequent publication.

## CONCLUSION

A method has been developed for the extraction and quantitation of the *cis*- and *trans*-isomers of clomiphene in plasma. The use of an on-line derivatisation by UV irradiation, coupled with fluorescence detection has enabled determination of clomiphene levels below 1 ng/ml of plasma. The sensitivity and selectivity of the technique will permit investigation of the pharmacokinetics of clomiphene.

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