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Note

Application of clomiphene photolysis to assays based on analysis of the derived phenanthrenes

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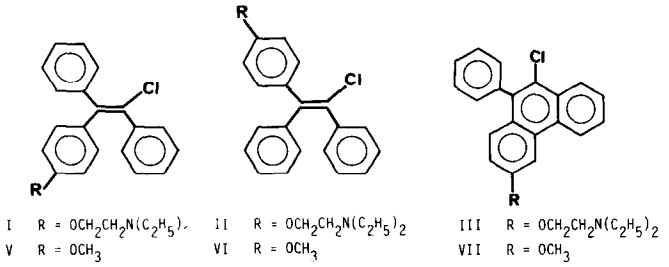
Clomiphene citrate, available commercially as a mixture of *E* (I) and *Z* (II) isomers, is used extensively for the treatment of female infertility. Although its ovulation-inducing properties were first reported in 1961[1], definitive evidence on its exact mode of action has only recently been cited[2]. Despite being in use for some 20 years, only one assay for clomiphene has been published which is based on HPLC high-performance liquid chromatography (HPLC)–post-column photolysis–fluoresence detection[3]. This method is an adaption of assays previously designed for tamoxifen (X), which has the same basic triphenylethylene skeleton[4,5]. These assays rely on the well documented photocyclization of stilbenes to the corresponding fluorescent phenanthrene derivatives[6,7].



I R = $OCH_2CH_2N(C_2H_5)_2$
V R = OCH_3
II R = $OCH_2CH_2N(C_2H_5)_2$
VI R = OCH_3
III R = $OCH_2CH_2N(C_2H_5)_2$
VII R = OCH_3
IV R = $OCH_2CH_2N(C_2H_5)_2$
VIII R = OCH_3
IX R = OCH_3
X R = $OCH_2CH_2N(CH_3)_2$

A limitation of the reported clomiphene assay is both the lack of an internal standard and the need to maintain constant conditions for transit-time with respect to the post-column photolysis. These shortcomings could be potentially resolved by photocyclization of clomiphene prior to HPLC analysis. The actual conversion of

clomiphene to the phenanthrenes III and IV has not been investigated, although Adam *et al.*[5] have noted that the fluorescent yield for clomiphene appeared to be considerably lower than that for tamoxifen (X).

EXPERIMENTAL

Reagents

Clomiphene citrate, and the pure individual *Z* and *E* isomers were donated by Merrel Dow Pharmaceuticals (Sydney, Australia). HPLC assay (see below) established that *Z*-clomiphene (II) contained approximately 5% of the *E* isomer (II). The *E*- and *Z*-4-(2-chloro-1,2-diphenylvinyl)anisoles V and VI were available from previous studies in this laboratory[8]. Methenamine was prepared as described by Vogel[9]. Acetonitrile, dichloromethane, methanol and chloroform were purchased from Waters Assoc. (Sydney, Australia) and were redistilled prior to use.

Gas chromatography and mass spectrometry

Gas chromatographic (GC) separations were performed using a 12 m × 0.3 mm I.D. vitreous silica bonded phase column [BPI, Scientific Glass Engineering (SGE), Melbourne, Australia]. The instrument, a Pye 104 gas chromatograph (Pye Unicam, Cambridge, U.K.), was equipped with an on-column injector (Model OCl-3, SGE) and a flame-ionization detector. The normal GC conditions were as follows: initial column temperature 240°C (2 min), then 20°C/min to a final temperature of 275°C. Hydrogen (2 ml/min) was used as the carrier gas. The retention indices for compounds I–IV, measured at 240°C, are 2955, 3004, 3377 and 3413, respectively. The methoxy compounds V–VIII were analysed at 175°C.

Mass spectral analysis was performed on a Hewlett-Packard 5992 instrument (Hewlett-Packard, Palo Alto, CA, U.S.A.), in which the 12 m × 0.3 mm column was interfaced to the ion-source via an all-glass jet separator. Helium was used as the carrier gas.

High-performance liquid chromatography

For HPLC, a Waters instrument was used equipped with an universal liquid injector and a variable-wavelength UV detector set at 298 nm. Analyses were performed on a stainless-steel column (300 × 33 mm) containing a silica packing (μPorasil, 10 μm), purchased from Waters. The elution solvent was acetonitrile–dichloromethane (20:80) containing 0.1% (w/v) methenamine[10], at a flow-rate of 1.6 ml/min.

Photochemistry

A high-pressure mercury lamp (125 W) was used for all the photolytic reactions. Experiments were conducted at both high (3 m*M*) and low (0.1 m*M*) concentrations in the appropriate solvent. The studies at high concentration were performed in a photochemical reaction vessel containing an immersion sleeve for the lamp. In some experiments iodine was added at a molar concentration approximately 20–30 times lower than that of the compound of interest. The low-concentration reactions were conducted in quartz cuvettes placed in a box container into which the lamp was lowered. Each cuvette was located so that it was approximately equidistant from the UV source.

RESULTS AND DISCUSSION

Whilst HPLC, in comparison to GC, allowed both complete baseline resolution and increased sensitivity for the clomiphene isomers I and II, the phenanthrenes III and IV could only be separated by GC (Fig. 1). Photolysis of either V or VI yielded one phenanthrene product, by either HPLC or GC, which was assumed to be a mixture of compounds VII and VIII. The mass spectrum for the VII/VIII chromatographic peak showed an intense molecular ion at *m/z* 318, confirming the structural assignment (Fig. 2). The standard HPLC conditions employed could not separate compounds V and VI. Due to the difficulty in analysing isomer ratios for the methoxy system V/VI, application of these compounds was mostly restricted to assess side-reactions in the photochemical process. The electron-impact mass spectra for the phenanthrenes III and IV are shown in Fig. 3. While the methoxy compounds VII/VIII show an intense molecular ion, spectra for compounds I–IV, possessing the $(C_2H_5)_2NCH_2CH_2O$– side-chain are dominated by its respective cleavage to generate the $[(C_2H_5)_2N{=}CH_2]^+$ fragmentat *m/z* 86, and the aziridine homologue, $[(C_2H_5)_2NCH_2CH_2]^+$ at *m/z* 100. Definitive structural assignment for the two phenanthrenes III and IV is not possible without synthesis of authentic samples or their isolation and subsequent characterization by nuclear magnetic resonance spectrometry. However, a tentative characterization is possible based on information reported for authentic phenanthrene samples in the tamoxifen study of Adam *et al.*[5].

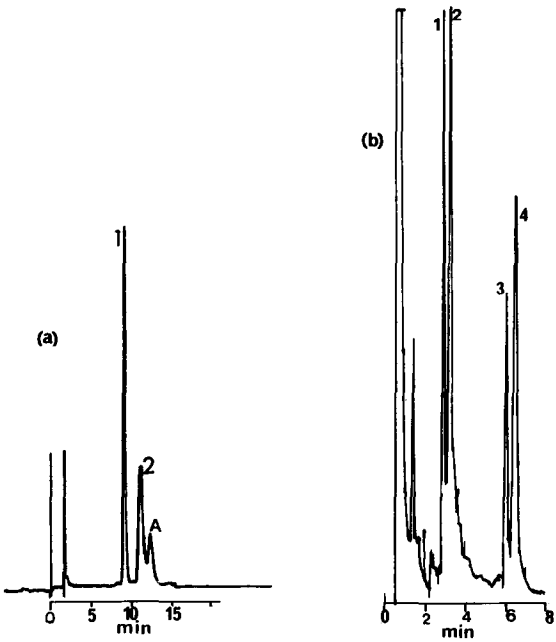


Fig. 1. (a) HPLC chromatogram, after 5 min of photolysis of *E*-clomiphene at 0.1 m*M* in chloroform. Conditions as given in the Experimental section; 20 μl of reaction mixture injected. (b) GC chromatogram, after 15 min of photolysis of *Z*-clomiphene at 0.1 m*M* in cloroform. Initial temperature 240°C (4 min), then 20°C/min to 275°C. Reaction sample concentrated by a factor of twenty and 1 μl injected. Peaks: 1 = *E*-clomiphene (I); 2 = *Z*-clomiphene (II); 3 = phenanthrene III; 4 = phenanthrene IV; A = mixture of phenanthrenes III and IV.

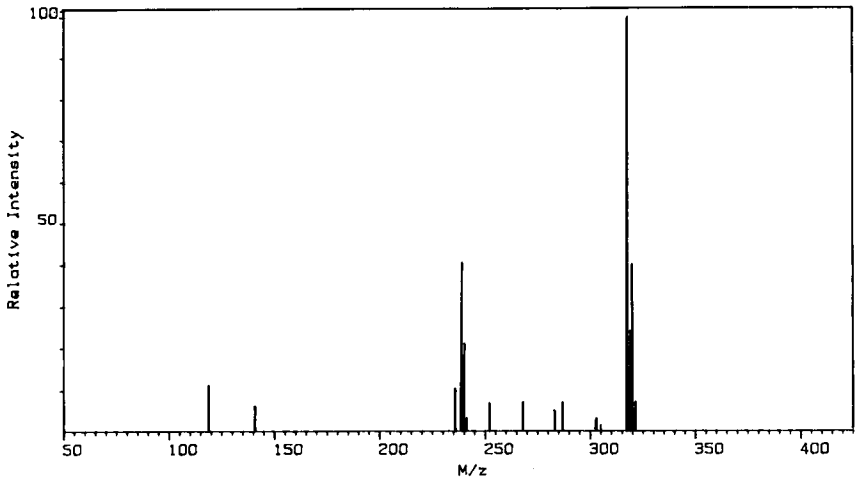


Fig. 2. Electron-impact mass spectrum for methoxyphenanthrene (VII/VIII), mol.wt. 318. Spectra recorded by GC–mass spectrometry using conditions described in the Experimental section.

Firstly, the phenanthrene with the side-chain located on the actual phenanthrene skeleton was found by GC (1% OV-1, 240°C) to have a shorter retention time. Secondly, the ratio of the side-chain fragments, $[(CH_3)_2N=CH_2]^+$ (*m/z* 58) and $[(CH_3)_2NCH_2CH_2]^+$ (*m/z* 72) was significantly increased for the phenanthrene with the side-chain on the poly-aromatic ring system. The structures assigned as III and IV, respectively, are seen to reflect these facts. The phenanthrene III has a lower retention index and an increased ratio of *m/z* 86 to *m/z* 100 (Fig. 3).

Investigation of the photochemistry of clomiphene and its analogues established the following facts. The formation of side-products is solvent dependent. In methanol, a mixture of unknown products is formed with minimal yield of the desired phenanthrenes. Use of chloroform or dichloromethane however, leads to predominant formation of the phenanthrenes. The major side-reaction, which is only apparent at the higher concentration (3 m*M*), is oxygen addition across the double bond, followed by scission to generate the respective substituted benzophenone IX. The mass spectrum for the latter compound is shown in Fig. 4. At dilute concentrations of the reactant in chloroform–dichloromethane the reaction rate is not markedly increased by the addition of iodine[7].

With respect to the actual clomiphene photolysis reaction, it was found that isomerization between compounds I and II occurs concomittantly to the formation of phenanthrenes. Therefore, photolysis of either *E*-clomiphene (I) or *Z*-clomiphene (II), and calculation of the ratios of unreacted I and II with respect to time, gave the results summarised in Table I. The photostationary ratio of I:II of 1.0 correlates with the similar extinction coefficients for the two isomers[11,12]. At completion of the reaction the ratios of the phenanthrenes III:IV were 1.04 and 0.81, reflecting a bias towards the respective starting isomer I or II. The latter result is indicative of the isomerization–cyclization pathways operating during the photolytic process.

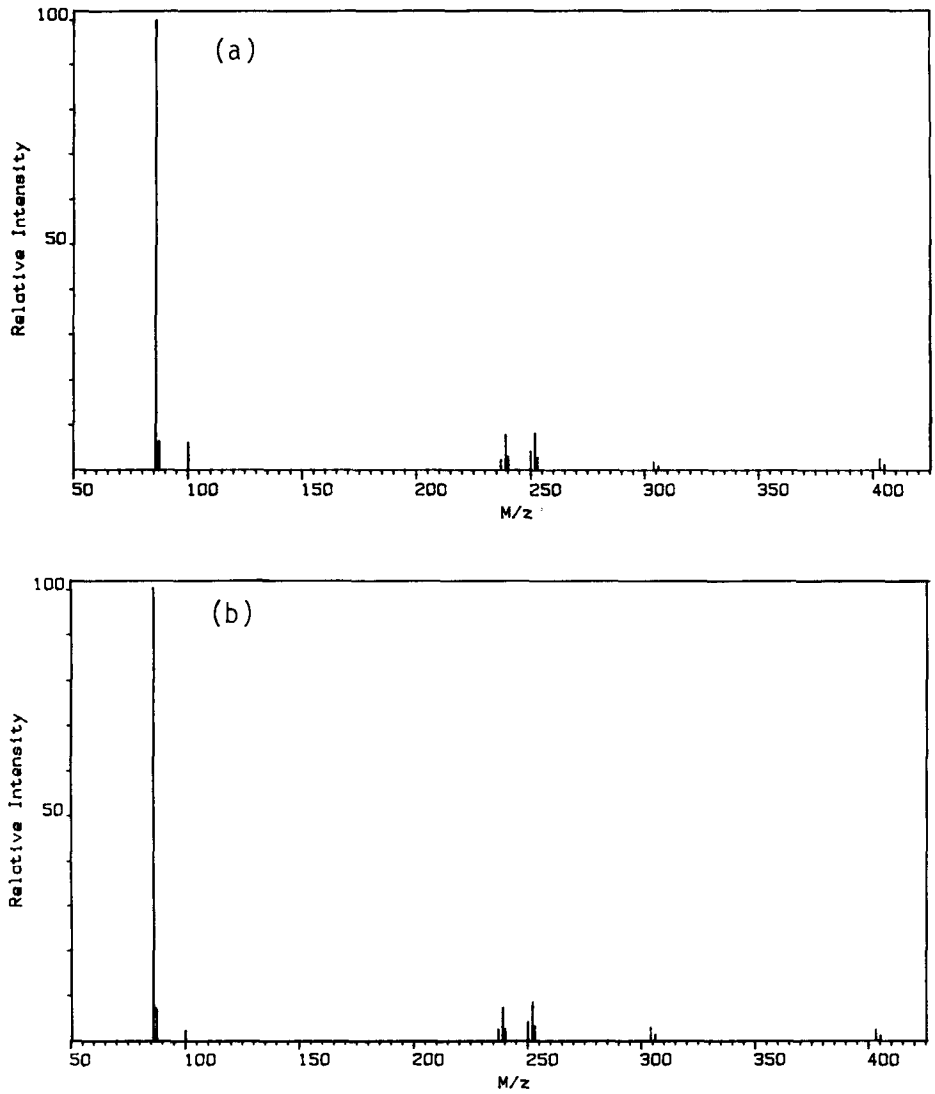


Fig. 3. Electron-impact mass spectra for the phenanthrenes III(a) and IV(b), mol.wt. 403. For information on the significance of the *m/z* 86 and 100 ions see the text.

This study has therefore established that quantitation of the clomiphene isomers I and II by photolysis and estimation of the respective phenanthrenes III and IV, is not a viable analytical method. If photolysis is to be used, then it must be performed post-column, after resolution of the isomers I and II by HPLC, as previously described[3].

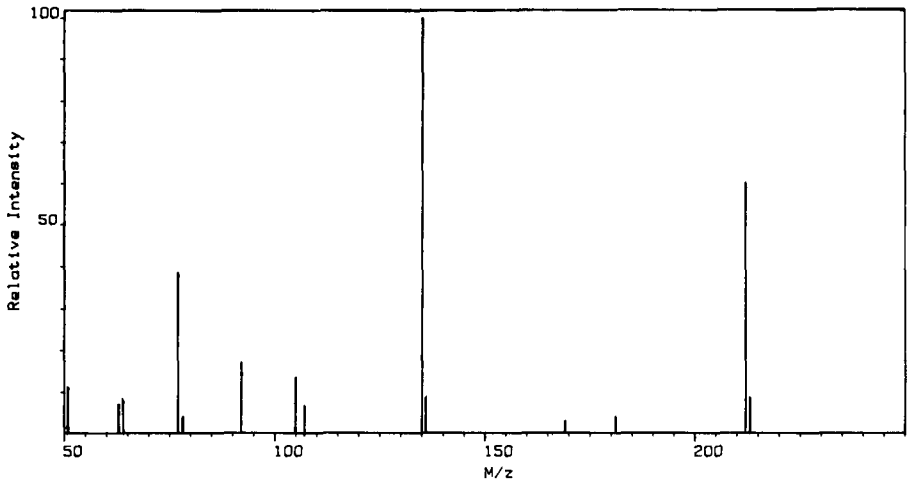


Fig. 4. Electron-impact mass spectra of major side-product, assigned the substituted benzophenone structure (IX). The major ion at m/z 135 represents the fragment $CH_3OC_6H_6CO^+$ formed by loss of the phenyl ring. The fragment at m/z 105, $C_6H_6CO^+$, is considerably less intense as predicted by detailed studies for this type of process[13].

TABLE I

RATIO OF *E*-CLOMIPHENE TO *Z*-CLOMIPHENE DURING PHOTOLYSIS IN CLOROFORM (0.1 m*M*)

Time (min)	*Starting compound*		
	E-Clomiphene	*Z-Clomiphene*	*Mixture*
0	–	0.05	2.18
5	3.33	0.42	1.42
10	1.50	0.66	1.40
15	1.09	0.82	1.14
30	1.00	1.00	0.97

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REFERENCES

1 R. B. Greenblatt, W. E. Barfield, E. C. Junget and A. W. Roy, *JAMA, J. Am. Med. Assoc.*, 178 (1961) 101.
2 J. F. Kerin, J. H. Liu, G. Phillipou and S. S. C. Yen, *J. Clin. Endocr. Metab.*, 61 (1985) 265.
3 P. J. Harman, G. L. Blackman and G. Phillipou, *J. Chromatogr.*, 225 (1981) 131.
4 D. W. Mendenhall, H. Kobayashi, F. M. L. Shih, L. A. Sternson, T. Higuchi and C. Fabian, *Clin. Chem.*, 24 (1978) 1518.

5 H. K. Adam, M. A. Gay and R. H. Moore, *J. Endocr.*, 84 (1980) 35.
6 F. B. Mallory, C. S. Wood and J. T. Gordon, *J. Am. Chem. Soc.*, 86 (1964) 3094.
7 C. S. Wood and F. B. Mallory, *J. Org. Chem.*, 29 (1964) 3373.
8 D. W. Johnson, G. Phillipou and C. J. Seaborn, *Aust. J. Chem.*, 33 (1980) 461.
9 A. I. Vogel, *Practical Organic Chemistry*, Longmans, London, 3rd ed., 1964, p. 326.
10 M. S. F. Ross and H. Judelman, *J. Chromatogr.*, 298 (1984) 172.
11 A. Richardson, Jr., H. D. Benson and G. Hite, *J. Pharm. Sci.*, 65 (1976) 1545.
12 G. S. Hammond, J. Satiel, A. A. Lamola, N. J. Turro, J. S. Bradshaw, D. O. Cowan, R. C. Counsell, V. Vogt and C. Dalton, *J. Am. Chem. Soc.*, 86 (1964) 3197.
13 H. Budzikiewicz, C. Djerassi and D. H. Williams, *Mass Spectrometry of Organic Compounds*, Holden-Day, San Francisco, CA, 1967, p. 164.