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ANALYSIS OF DIETHYLSTILBESTROL AND ITS IMPURITIES IN TABLETS USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A. R. LEA, W. J. KAYABA and D. M. HAILEY
National Biological Standards Laboratory, P. O. Box 462, Canberra City, A.C.T. 2601 (Australia)
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SUMMARY

A high–performance liquid chromatographic (HPLC) method is described which is capable of resolving *cis*- and *trans*-diethylstilbestrol (DES), DES mono- and dimethyl ethers and 4,4′-dihydroxystilbene. The mobile phase and internal standard used stabilise the *cis–trans* DES isomer ratio, and the method is capable of quantitating both isomers in dosage forms without derivatisation. Recovery of DES from tablets is quantitative. Results of tablet analyses using this method are compared with those obtained with the official spectrophotometric procedure.

INTRODUCTION

Diethylstilbestrol (DES) or 3,4-bis-(4-hydroxyphenyl)hex-3-ene is a non-steroidal estrogen which is used in the treatment of menopausal symptoms and of malignant neoplasms of the breast and prostate and has also been used as a post-coital contraceptive. The *trans* isomer is used for therapeutic purposes, as early evidence was obtained that the *cis* isomer had much less estrogenic activity[1]. The difference in activity has been related to the ability of the *trans* isomer to mimic the stereochemical configuration of the steroid ring system[2]. Present pharmacopoeial methods for assay of DES in tablets[3,4] are of limited specificity, depending on the photodegradation of *trans*-DES to a tricyclic chromophore. This photodegradation entails the *in situ* conversion of the *trans* to the *cis* isomer[5], so that it is not possible to determine the content of the less active compound. In addition to this problem, the present official method for Australia (BP)[3], which uses dehydrated alcohol to extract the active substance, gives low recoveries of DES from some formulations. Hussey *et al.*[6] found that the use of water in the extraction process was necessary for complete recovery of DES. The work reported here was initiated as a result of an apparent failure of a DES tablet formulation tested as part of a routine sampling program. The content of active substance as measured by the BP procedure was only 86% of label claim, which is below specification. Checks carried out using an alternative extraction procedure (USP) and a high-performance liquid chromato-

graphic (HPLC) separation gave higher recoveries and revealed the presence of an impurity which was thought to be the *cis* isomer. It was therefore decided to develop a method which would be capable of detecting both isomers in preparations containing DES.

Some of the previous attempts to analyse both *trans*- and *cis*-DES were unsuccessful because of the propensity for these compounds to form an equilibration mixture under certain conditions. Isolation of the *cis* compound failed because of rapid isomerisation to the *trans* isomer. In addition, the earlier literature on these compounds is confused because of the presence of the isomer 3,4-bis-(4-hydroxyphenyl)hex-2-ene or *pseudo*-DES in the reaction product[1,2]. It is only relatively recently that the *cis* isomer has been isolated in high purity by White and Ludwig[7]. These authors reported studies on the effects of solvent and temperature on the *cis*–*trans* isomerisation process. Solvents such as chloroform, benzene and ether enhanced the rate of isomerisation while much slower rates were reported in dimethylsulphoxide, dimethylformamide and ethanol. Winkler *et al.*[8] prepared 90% pure *cis*-DES disodium salt by the separation of the *cis* and *trans* diacetates on preparative thin-layer chromatography (TLC) followed by hydrolysis to form the sodium salts. Kinetic studies of the isomerisation in the ionised and non-ionised forms were undertaken and a bimolecular mechanism was proposed for isomerisation of the non-ionised forms. Isomerisation is inhibited in solvents such as ethanol because of hydrogen bonding of the solvent to the phenolic groups in DES.

As a result of the use of DES as a growth promoter in livestock food mixes, several methods exist for the analysis of DES in animal foods, premixes and animal tissue. A number of these methods[9–18] involve the use of gas–liquid chromatography (GLC), but most are not concerned with the separate quantitation of the isomers. Rutherford[10] achieved a separation of the *cis* and *trans* isomers as the trimethylsilyl ethers, and these derivatives were found not to isomerise. Fontani and Cova[12] reported a GLC separation of the trifluoroacetyl esters of the *cis*, *trans* and *pseudo* isomers on a QF-1 column and also a preparative TLC procedure for these compounds. Diethylstilbestrol and its monomethyl ether have also been separated on a QF-1 column as the trimethylsilyl derivatives[16].

Roos[5] described an HPLC separation of the photodegradation products of DES. This included a partial separation of the *cis* and *trans* isomers and the method was also applied to DES creams, tablets and suppositories using phenothiazine as an internal standard. However, no mention was made of analysis of 4,4′-dihydroxystilbene and the mono- and dimethyl ether impurities. Tests for these compounds are included in the relevant monograph of the BP (stilboestrol), for DES substance. In addition, detection of small quantities of *cis*-DES would be difficult because this compound was only partially resolved from the *trans* isomer. *cis*-DES was not detected in the samples. King *et al.*[19] described the use of a liquid–solid HPLC system to resolve the isomers of DES. A good separation of *cis*- and *trans*-DES was achieved and the system was applied to the analysis of DES in animal chow. The sample preparation produced a 1:4 mixture of *cis* and *trans* isomers regardless of original sample composition. This aspect of DES analysis is common to all the *in vivo* animal studies with DES and no method has been reported to determine the amount of isomerisation *in vivo*. On the basis of the information available in the literature, it seemed appropriate to investigate the use of HPLC for the analysis of both DES

substance and formulations. The requirements for the method were that it should not cause interconversion of *cis* and *trans*-DES and that it should be capable of resolving these isomers and the impurities cited in the monograph on stilboestrol of the BP 1973.

EXPERIMENTAL

Materials

trans-DES was obtained from BDH Pharmaceuticals (Vic., Australia). Mono- and dimethyl ethers of DES were BP reference substances and 4,4'-dihydroxystilbene was obtained from K & K Labs. (ICN) (Plainview, N.Y., U.S.A.). Chloroform and methanol were analytical reagent grade (Merck, Darmstadt, G.F.R.), 4-chloro-*m*-cresol was purchased from BDH Chemicals (Poole, Great Britain) and trifluoroacetic anhydride (TFA) and N,O-bis(trimethylsilyl)-acetamide (BSA) were supplied by Pierce (Rockford, Ill., U.S.A.).

Water used in the HPLC mobile phase was freshly distilled.

Apparatus

Liquid chromatograph. A Varian 4100 high-pressure liquid chromatograph with a Varian 635D spectrophotometer as the detection unit was used.The spectrophotometer was fitted with an 8-μl flow cell and operated at 254 nm. The column was a Waters Associates μBondapak C_{18} (30 cm $\times$ 4 mm) and samples were introduced onto the column using an Altex 20-μl loop injector.

Gas chromatograph. A Perkin-Elmer Model 881 was used fitted with a 6 ft. $\times$ 1/8 in. glass column packed with 2.5% OV-101 on 80–100 mesh Gas-Chrom Q.

Calorimeter. A Perkin-Elmer DSC-18 was used for differential scan calorimetry and was calibrated at 429 °K with indium while the scan rate was 8°/min.

Spectrophotometer. Ultraviolet irradiation and spectrophotometric analysis were carried out in accordance with the method of the BP 1973[3] using a Chromatavue lamp and a Perkin-Elmer Model 124 spectrophotometer.

Preparation of standards

A standard mixture of *cis* and *trans*-DES was prepared by the method of Rutherford[10], by dissolving *trans*-DES in chloroform and allowing the isomerisation to equilibrate. This mixture was checked using Rutherford's method by GLC of the trimethylsilyl derivatives formed by reaction with BSA. The equilibration mixture was also checked by the method of Fontani and Cova[12]. Only two peaks were observed and it was assumed that only the *cis* and *trans* isomers were present. The TLC method of Fontani and Cova[12] was also used but no *pseudo*-DES was detected. Differential scan calorimetry when performed on the mixture gave a melting point of 142 °C for *trans*-DES.

Preparation of pure *cis*-DES by the method of White and Ludwig[7] was attempted but only low proportions of this isomer were obtained in the reaction mixture. A solution containing *cis*-DES with an isomeric purity of 90% was obtained by preparative HPLC carried out by Waters Assoc.

Chromatographic conditions

Mobile phase: methanol–water (75:25); pressure: 80 kg cm^{-2}; and detector: 254 nm, 0.5 a.u.f.s. attenuated through the recorder.

Internal standard solution

An amount of 3.6 g of 4-chloro-*m*-cresol was dissolved in 1000 ml of methanol–water (75:25).

Assay procedure

Twenty tablets were weighed accurately and ground to a fine powder. An amount equivalent to approximately 10 mg of DES was weighed into a 100-ml volumetric flask. The powder was extracted with 80 ml internal standard solution for 30 min with vigorous shaking. The solution was then made up to volume with internal standard solution. The resulting solution was filtered and the filtrate injected onto the column.

Eleven samples of DES tablets were assayed by this procedure and the results compared with those obtained using the method of the BP 1973.

Recovery experiments

The samples for the recovery analyses were made up as single tablets using the following formulation: lactose, 327.8 mg; wheat starch, 61.3 mg; gum acacia, 8.5 mg; magnesium stearate, 2.3 mg. DES, weighed individually into the excipients, nominal weight 10.0 mg.

After the addition of DES, the tablet mixture was shaken vigorously in a sealed vial. The mixture was then compressed in a hydraulic press using a pressure of 23 MPa. The resultant tablets were weighed and the amount of available DES recalculated by allowing for the losses in the compression process. The tablets were assayed by the procedure described above except that the tablets were crushed inside the volumetric flasks by the application of a glass rod.

cis-Diethylstilbestrol sensitivity factor

0.5 g of *trans*-DES was dissolved in chloroform and the *cis–trans* equilibration allowed to take place. Chloroform was evaporated off and the resulting mixture dried at 80° C. Samples of approximately 10 mg were weighed out and dissolved in internal standard solution and then assayed for *trans* isomer. The *cis* isomer content was determined by difference and the response factor calculated. The value of the response factor was found to be 1.8.

Precautions were taken to exclude light from the solution in all the assays and a fresh DES standard was made up in each new internal standard solution.

RESULTS AND DISCUSSION

Using the chromatographic system described above, *trans*-DES was resolved from *cis*-DES, *trans*-DES mono- and dimethyl ethers and 4,4′-dihydroxystilbene and this separation is shown in Fig. 1. The solubility data[7] for *cis*-DES suggest that this compound should elute before *trans*-DES in a reversed-phase system, as the *cis* isomer is the more soluble in polar solvents. However, as can be seen in Figs. 1

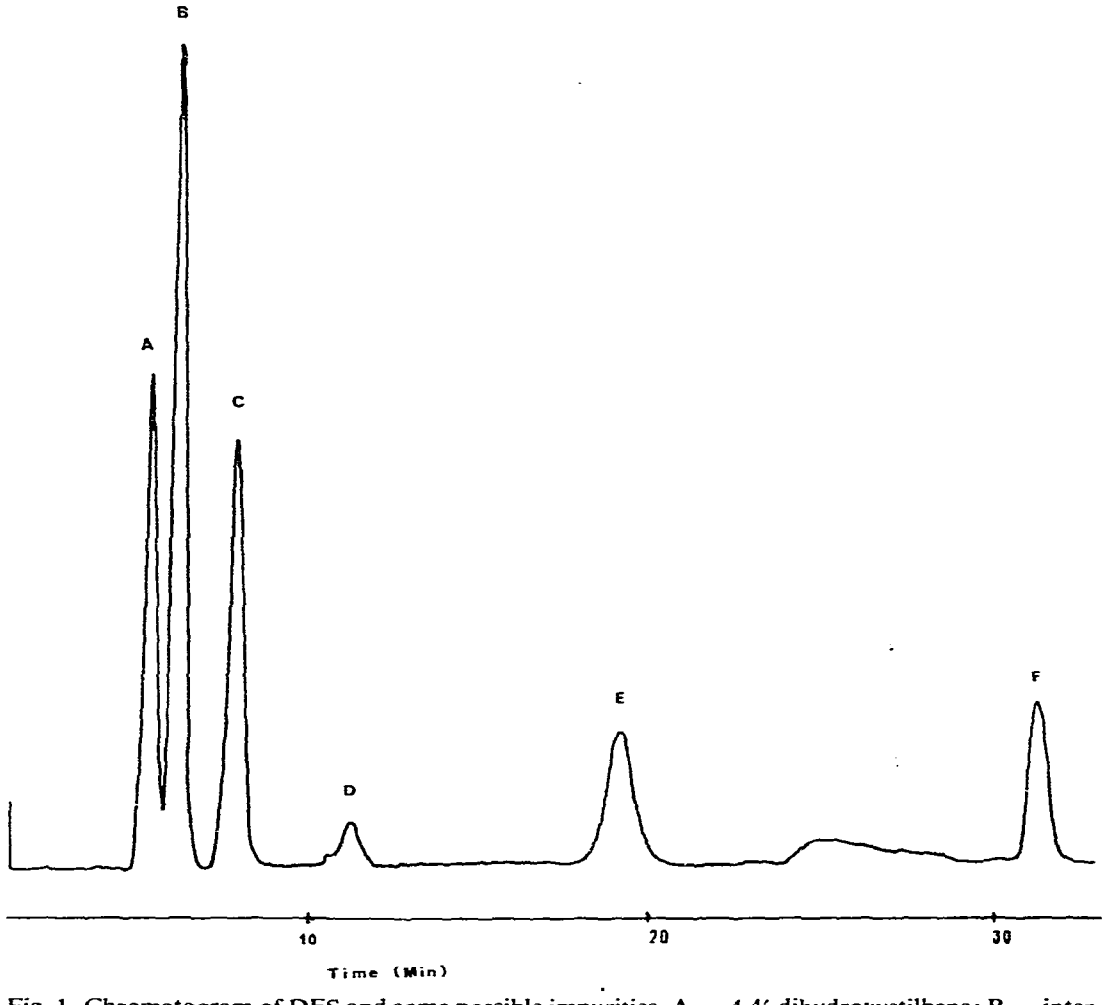

Fig. 1. Chromatogram of DES and some possible impurities. A = 4,4′-dihydroxystilbene; B = internal standard (4-chloro-*m*-cresol); C = *trans*-DES; D = *cis*-DES; E = *trans*-DES monomethyl ether; F = *trans*-DES dimethyl ether.

and 2, the *cis* isomer elutes after the *trans* isomer. This behaviour could be due to the fact that *cis*-DES can partition with the C_{18}-bonded phase while maintaining its polar phenolic functions at the interface or in the mobile phase. This cannot be achieved as readily by the *trans* isomer.

The choice of a reversed-phase system does much to stabilise the isomers in solution as the rate of isomerisation is much less in polar solvents[7]. King *et al.*[19] reported a rate of isomerisation in ethanol of 3% in 144 h. The rate observed in this study using methanol–water (75:25) was of the same order. The stabilisation of *trans*-DES with phenolic antioxidants has been reported[20] so that the choice of 4-chloro-*m*-cresol as an internal standard should further stabilise the isomer ratio. This stabilisation makes possible an accurate method of analysing for both *cis*- and *trans*-DES without derivatisation.

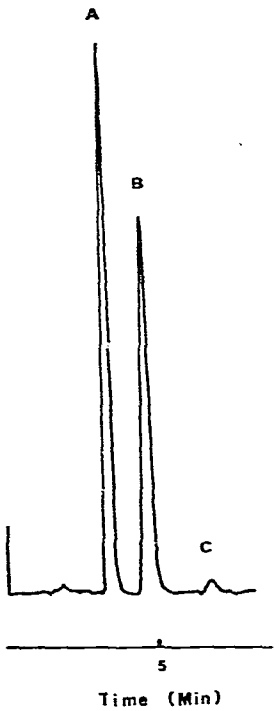

Fig. 2. Chromatogram from the assay of a sample of 10-mg DES tablets. A = internal standard; B = *trans*-DES; C = *cis*-DES.

TABLE I

ASSAY RESULTS FOR DES TABLET SAMPLES

The results for *trans*- and *cis*-DES are the means and standard deviations from four determinations. The BP assay results are the means from duplicate assays.

Sample	*trans-DES (%)*	*cis-DES (%)*	*Total DES (%)*		*I — II*
			HPLC (I)	*BP assay (II)*	
A	102.9 ± 1.2	4.2 ± 0.5	107.1	103.4	3.7
B	101.6 ± 3.9	ND*	101.6	99.3	2.3
C	98.2 ± 1.5	ND	98.2	99.0	−0.8
D	91.5 ± 1.9	ND	91.5	96.5	−5.0
E	97.3 ± 0.9	2.7	100.0	98.6	1.4
F	85.4	9.4	94.8	86.0	8.8
G	88.4 ± 1.3	5.6 ± 0.1	93.6	90.2	3.4
H	94.0 ± 0.8	ND	94.0	96.6	−2.6
I	94.5 ± 1.3	7.4 ± 0.4	101.9	105.9	−4.0
J	100.0 ± 9.6	ND	100.0	101.3	−1.3
K	86.7 ± 0.7	10.4 ± 0.2	97.1	96.8	0.3

* ND = Not detected.

The results of the assays of eleven tablet formulations appear in Table I. The HPLC results for the content of each isomer are shown as the means and standard deviations of four assays. The results obtained using the BP method are shown

as the mean of duplicate assays. Six of the eleven samples contained measurable quantities of the *cis* isomer. Agreement between the HPLC and spectrophotometric (BP) results was reasonably good except for samples D and F. Sample F was the disputed sample mentioned above which initiated this work; the low result using the BP assay is thought to be due to low recovery in the extraction stage. In the case of sample D, the higher result with the BP method may indicate interference by an excipient or the presence of an artifact formed in the photodegradation step. There is no significant difference between the assay results from the two methods at the 95% confidence level (paired *t*-test). A typical chromatogram from one of the tablet assays is shown in Fig. 2.

The results of the recovery experiments are shown in Table II. The mean recovery for the eight samples was 100.8% with a coefficient of variation of 0.9%. Extraction of DES by this method is therefore quantitative. The response of the UV detector to DES was found to be linear over the range 0.2–2.0 μg/ml.

TABLE II

RESULTS OF RECOVERY EXPERIMENTS

Mean recovery 100.8 ± 0.9%.

Sample	*Expected amount (mg)*	*Amount found (mg)*	*Recovery (%)*
1	9.68	9.88	102.1
2	8.35	8.42	100.8
3	9.53	9.51	99.8
4	7.23	7.36	101.8
5	11.16	11.24	100.7
6	10.18	10.24	100.6
7	11.74	11.87	101.1
8	10.42	10.34	99.2

The HPLC method described here compares favourably with the official (BP) assay procedure, being quicker, capable of quantitating both *trans*- and *cis*-DES and several potential impurities and overcoming possible recovery problems by use of an extraction system which includes water. The HPLC procedure is also suitable for single-tablet assay for tests for the uniformity of content. The presence of *cis*-DES in a number of commercially available formulations is of interest and it may be appropriate to set a limit for the permitted content of this isomer in future official standards. The value of this limit will depend on the pharmacological activity of *cis*-DES. This is reported to be less than that of the *trans* isomer[9,21,22], but the extent of the activity difference is still unclear. Dodds[21] has reported that the *cis* compound has about one twentieth of the estrogenic activity of *trans*-DES. However, Winkler *et al.*[8] found that the *cis* isomer showed approximately 45% of the activity of the *trans* compound. These data are in conflict with the data for the *cis* and *trans* esters, where the *trans*-DES dipropionate demonstrated 600 times more activity than the corresponding *cis* isomer. The authors[8] attribute the enhanced activity of the *cis*-DES over the *cis*-DES ester to possible *in vivo* isomerisation of the unesterified

isomer. Application of the HPLC method to this problem should make it possible to measure the extent of *cis* to *trans* conversion *in vivo* and provide a better understanding of the pharmacokinetic profiles of these compounds.

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