Journal of Chromatography, 344 (1985) 378–384
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2732

Note

Separation and detection of enantiomers of stilbestrol analogues by combined high-performance liquid chromatography—thermospray mass spectrometry

C.E. PARKER*, L.A. LEVY, R.W. SMITH, K. YAMAGUCHI* and S.J. GASKELL**

Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709 (U.S.A.)

and

K.S. KORACH

Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709 (U.S.A.)

(First received February 25th, 1985; revised manuscript received June 12th, 1985)

The synthetic estrogen, diethylstilbestrol [DES; *E*-3,4-bis(*p*-hydroxyphenyl)-hex-3-ene] has been widely used in human medicine and as a growth-promoting agent in livestock. The discovery of an association between human gestational use and an increased incidence of a rare vaginal cancer in daughters [1], and of genital tract abnormalities in sons [2] has directed attention towards detailed study of the metabolism and mechanism of action of DES.

Oxidative metabolism studies have indicated a number of products [3], with varying affinities of binding to the estrogen receptor [4]. Biochemical [5] and X-ray crystallographic [6] studies of these DES compounds have indicated that no clear correlation exists between structure and activity. The importance of stereochemical features is indicated by studies such as those of Landvatter and Katzenellenbogen [7], who demonstrated differences in estrogen receptor binding between enantiomeric derivatives of norhexestrol

*On leave from Showa University, Tokyo, Japan.
**On leave from the Tenovus Institute for Cancer Research, University of Wales College of Medicine, Cardiff, U.K.

[2,3-bis(*p*-hydroxyphenyl)pent-2-ene]. Such results emphasize the need for effective techniques for the separation and identification of stereoisomers, including enantiomers, in investigations of structure—activity relationships for DES metabolites and analogues.

Recent developments in high-performance liquid chromatography (HPLC) using chiral phases have provided a convenient approach to enantiomer separation, while the coupling of HPLC and mass spectrometry (MS) has given a means for sensitive detection and identification. Crowther et al. [8], for example, recently reported analyses of enantiomeric carboxylic acid derivatives using direct liquid introduction HPLC—MS. Here we report the analysis of enantiomers of indenestrol A (a metabolite of DES) and indenestrol B using HPLC—thermospray MS.

EXPERIMENTAL

Samples and solvents

Samples of *E*-diethylstilbestrol (E-DES: 3,4-bis(*p*-hydroxyphenyl)-3-hexene), *meso*-hexestrol (*meso*-3,4-bis(*p*-hydroxyphenyl)-*n*-hexane), and *E,E*-dienestrol (3,4-bis-(*p*-hydroxyphenyl)-2,4-hexadiene) were obtained from Sigma (St. Louis, MO, U.S.A.). Indenestrol A (1-ethyl-2-(4-hydroxyphenyl)-3-methyl-5-hydroxyindene), indenestrol B (1-methyl-2-(4-hydroxyphenyl)-3-ethyl-6-hydroxyindene) and *Z,Z*-dienestrol (3,4-bis(*p*-hydroxyphenyl)-2,4-hexadiene) were gifts from Dr. Manfred Metzler (University of Würzburg, Würzburg, F.R.G.).

HPLC solvents used in this study were HPLC-grade methanol (Fisher Scientific, Fair Lawn, NJ, U.S.A.), and water (MCB Reagents, FM Industries, Gibbstown, NJ, U.S.A.). The ammonium acetate used for the buffer was also from Fisher Scientific.

Instrumentation

The overall system diagram is shown in Fig. 1. The HPLC system consisted of three Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pumps, a Waters U6K injector, a Waters T-union, a Rheodyne Model 7010 injector (Rheodyne, Cotati, CA, U.S.A.) and a Waters Model 440 UV detector. The UV detector was connected to a Hewlett-Packard 3390A integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.).

The mass spectrometer used in these experiments was a VG 12-250

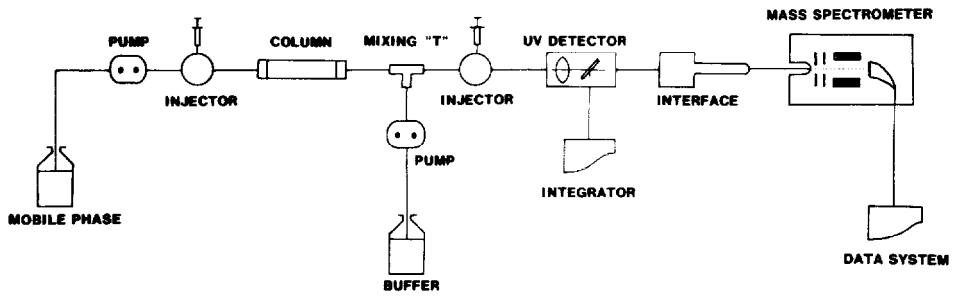

Fig. 1. Diagram of HPLC—MS system.

quadrupole mass spectrometer and data system, equipped with a VG thermospray interface (VG Masslab, Altrincham, U.K.). Thermospray probe temperatures were typically: vaporizer 400°C and desolvation heater 230°C (indicated). Source conditions were typically: temperature 200°C and VG Focus 4 electrode 250 V. The mass spectrometer was scanned from *m/z* 80 to 350 at 1 sec per scan.

The HPLC column used for the separations was a Jasco Chiralpak OT(+) column [9], 25 cm × 4.6 mm I.D. (Jasco, Easton, MD, U.S.A.). The column packing consists of a helical polymer of triphenylmethyl methacrylate, with chirality due to the helicity [9]. The column was operated at a flow-rate of 0.5 ml/min, the mobile phase was methanol—water (90:10). In order to prevent possible degradation of the column, the buffer required for thermospray ionization was added post-column (flow-rate 0.5 ml/min, 0.25 *M* ammonium acetate). Enantiomer separations were also attempted on a Pirkle Type 1A column (Regis Chemical, Morton Grove, IL, U.S.A.), consisting of (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine coated on silica [10]. All separations were performed at room temperature.

RESULTS AND DISCUSSION

Chromatograms resulting from UV detection (254 nm) and reconstructed-ion chromatograms (RIC) from the on-line mass spectral analysis of E-DES, the enantiomers of indenestrol A and B, the mixture of the geometrical isomers of

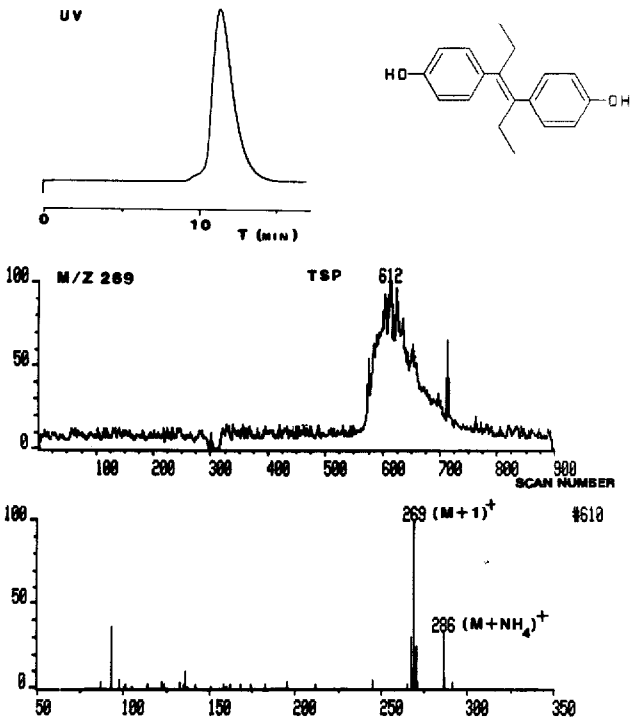

Fig. 2. UV trace, RIC trace, and mass spectrum of *E*-diethylstilbestrol.

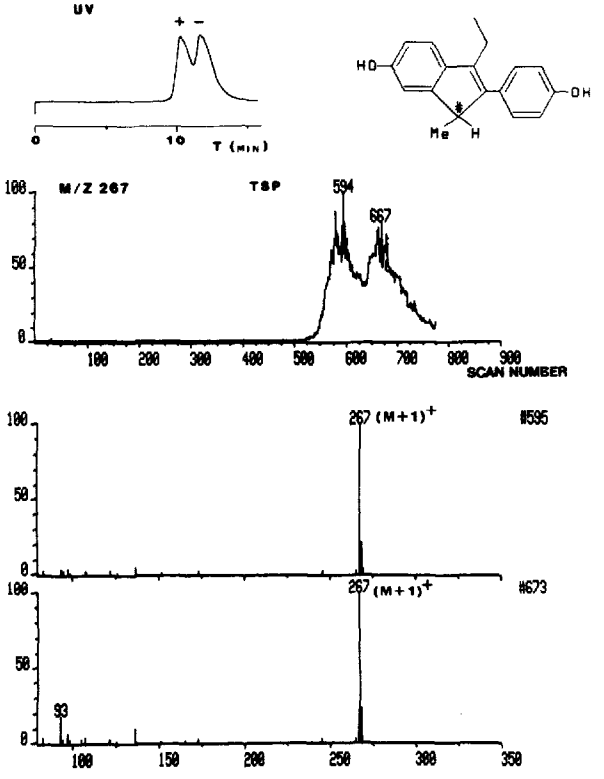

Fig. 3. UV trace, RIC trace, and mass spectra of enantiomers of indenestrol A.

E,E- and *Z,Z*-dienestrol, and *meso*-hexestrol are shown in Figs. 2—6, respectively.

HPLC separations

Attempts to separate the enantiomeric pairs on the Pirkle column were unsuccessful. Use of the Chiralpak column, however, yielded clear resolution of the enantiomers of both indenestrol A and B. The separations were confirmed by collecting the separated components and determining that (1) they possessed opposite optical rotation and (2) the NMR and direct-probe electron-impact mass spectra of the two peaks were identical.

It has been reported [11] that it is possible to add buffer post-column, analogous to post-column derivatization [12], in order to achieve the conditions necessary for ionization to occur during thermospray MS. No degradation of chromatographic resolution was observed, either during the buffer addition stage or in the thermospray interface itself, as can be seen from a comparison of the UV chromatograms and the RIC traces for the separated enantiomers of indenestrol A and B and from the geometrical isomers *E,E*- and *Z,Z*-dienestrol (Figs. 3—5, respectively).

Simple mass spectra were observed for all of the analytes, except for hexestrol, which was not detected even at levels in excess of 1 mg. The indenestrols and dienestrols yielded only MH^+ ions (Figs. 3—5) whereas [M +

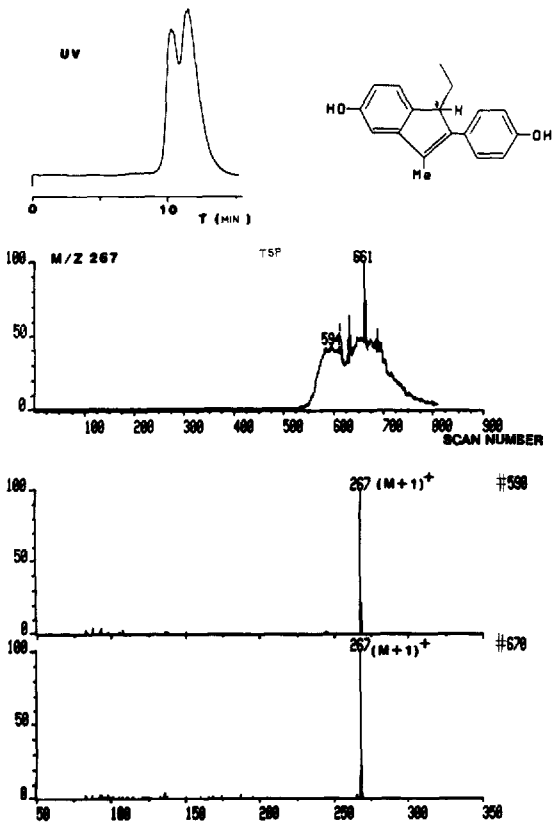

Fig. 4. UV trace, RIC trace, and mass spectra of enantiomers of indenestrol B.

$NH_4]^+$ was also present in the spectrum of E-DES (Fig. 2). The order of relative sensitivities was: indenestrol A and B $\cong$ *E,E*- and *Z,Z*-dienestrol > E-DES >>> hexestrol. It was estimated that a full-scan mass spectrum could be obtained with approximately 300 ng DES and 3 ng dienestrol.

Vestal and co-workers [13, 14] have suggested that compounds not ionized in solution generate protonated molecular ions during the thermospray process by a chemical-ionization reaction in the high-pressure region near the thermospray probe tip, i.e. in a gas phase process. In this region, NH_4^+ will be the principal protonating agent; accordingly, the observation of MH^+ ions is expected when the proton affinity of the analyte exceeds that of ammonia. The relative sensitivities given above parallel the expected order of proton affinities, reflecting the relation between proton affinity and extent of conjugation in the molecule [15].

Analyses of the same series of compounds were performed using a direct-insertion solids probe and conventional ammonia chemical ionization. The relative sensitivities of detection and the relative abundances of the MH^+ and $[M + NH_4]^+$ ions paralleled the observations made in the thermospray analyses.

CONCLUSIONS

The nature of the Jasco Chirapak (OT+) column allows the separation of

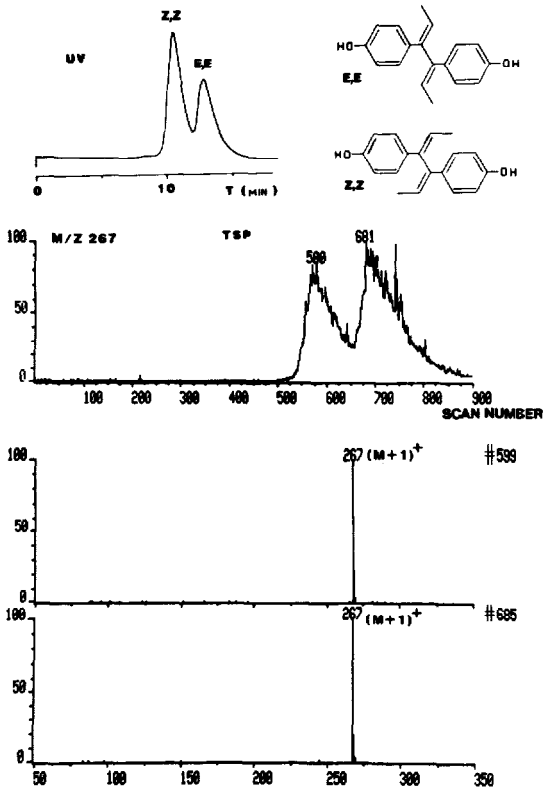

Fig. 5. UV trace, RIC trace, and mass spectra of *E,E*- and *Z,Z*,-dienestrol.

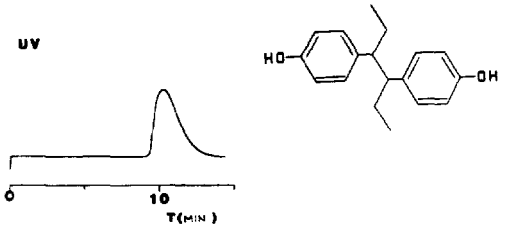

Fig. 6. UV trace of *meso*-hexestrol.

enantiomers. Analysis of biological fluids requires preliminary separation and collection of the different estrogens by another chromatographic technique, followed by the enantiomer separation. This additional step is required because the sensitivity of the Chirapak column precludes direct analysis of biological materials. In addition, restrictions on the polarity of the mobile phase result in similar retention times for the various stilbestrol analogues. However, this rapid means of separation and identification of enantiomers, an otherwise difficult process to carry out on a micro scale, allows the close examination of subtle structure—activity relationships [16].

In conclusion HPLC—thermospray MS using a column packed with a chiral stationary phase has been shown to be useful for on-line separation and MS analysis of E-DES, *E,E*- and *Z,Z*-dienestrol, and for the resolution and MS

analysis of the related chiral synthetic stilbestrol estrogens, indenestrol A and B.

REFERENCES

1 A.L. Herbst, D.C. Poskanzer, S.J. Robboy, L. Friedlander and R.E. Scully, N. Engl. J. Med., 292 (1975) 334.
2 W.B. Gill, G.F. Schumacher and M. Bibbo, J. Urol., 117 (1977) 477.
3 M. Metzler, CRC Crit. Rev. Biochem., 10 (1981) 171.
4 K.S. Korach, M. Metzler and J.A. McLachlan, Proc. Natl. Acad. Sci. U.S.A., 75 (1978) 468.
5 K.S. Korach, in W.W. Leavitt (Editor), Biochemical and Estrogenic Activity of Some DES Metabolites and Analogoues in the Mouse Uterus, Plenum Press, Baltimore, MD, 1982, pp. 39—62.
6 W.L. Duax, D.C. Swenson, P.D. Strong, K.S. Korach, J.A. McLachlan and M. Metzler, Mol. Pharmacol., 26 (1984) 520.
7 S.W. Landvatter and J.A. Katzenellenbogen, Mol. Pharmacol., 20 (1981) 43.
8 J.B. Crowther, T.R. Covey, E.A. Dewey and J.D. Henion, Anal. Chem., 56 (1984) 2921.
9 Y. Okamoto, I. Okamoto and H. Yuki, Chem. Lett., 7 (1981) 835.
10 W.H. Pirkle, J.M. Finn, J.L. Schreiner and B.C. Hamper, J. Amer. Chem. Soc., 103 (1981) 3694.
11 R.D. Voyksner, J.T. Bursey and E.D. Pellizzari, Anal. Chem., 56 (1984) 1507.
12 S. Moore, D.H. Spakman and W.H. Stein, Anal. Chem., 30 (1958) 1185.
13 M.L. Vestal, Int. J. Mass Spectrom. Ion Phys., 46 (1983) 193.
14 D. Pilosof, H.Y. Kim and M.L. Vestal, Proceedings of the 32nd Annual Conference on Mass Spectrometry and Allied Topics, San Antonio, TX, May 27—June 1, 1984.
15 A.G. Harrison, Chemical Ionization Mass Spectrometry, CRC Press, Boca Raton, FL, 1983, pp. 31—32.
16 K.S. Korach, L.A. Levy, and P.J. Sarver, in J.A. McLachlan (Editor), Stereochemical Analysis of Stilbene Estrogens: Receptor Binding and Hormone Responsiveness, Elsevier/North Holland, Amsterdam, in press.