Journal of Chromatography, 339 (1985) 87—95
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2469

APPLICATION OF A CAPILLARY GAS CHROMATOGRAPHIC—SELECTED-ION RECORDING MASS SPECTROMETRIC TECHNIQUE TO THE ANALYSIS OF DIETHYLSTILBESTROL AND ITS PHOSPHORYLATED PRECURSORS IN PLASMA AND TISSUES

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(First received May 28th, 1984; revised manuscript received November 16th, 1984)

SUMMARY

A method for the analysis of diethylstilbestrol (DES) which is suitable for pharmacokinetic studies has been developed using capillary gas chromatographic (GC) separation and detection with selected-ion mass spectrometry (MS). This technique has been applied to a variety of samples including human plasma samples and extracts of animal tissues including prostate and liver. To investigate the pharmacokinetics of stilphostrol (diethylstilbestrol diphosphate) we have modified the GC—MS method in two ways. One modification involves a dual assay for DES; the first a direct assay, and the second after hydrolysis of a sample with alkaline phosphatase. The difference in these values is the amount of phosphorylated DES present. The other modification separates stilphostrol and DES using a reversed-phase, ion-paired high-performance liquid chromatographic method followed by alkaline phosphatase hydrolysis followed by the GC—MS method. The details of these three methods are described and some representative data are shown.

INTRODUCTION

Diethylstilbestrol (DES) is widely publicized as an animal feed additive [1]. Because of its toxicological properties, there have been many methods developed for its analysis [2]. Few, if any, of these methods were used for pharmacokinetic studies — their foci were in the area of regulatory analyses. On the other hand, DES is a frequently used drug in treating prostatic cancer in man [3]. Although used for over 40 years, little effort has been spent on determining its clinical pharmacokinetics. Recently, Kemp et al. [4] used both gas chromatographic—mass spectrometric (GC—MS) and radioimmmunoassay methods to describe the blood levels following oral administration of DES in

0378-4347/85/$03.30

men with prostatic cancer. More recently, work from this laboratory [5] compared the bioavailability of DES in patients receiving either oral DES or intravenous stilphostrol (diethylstilbestrol diphosphate). That study also compared both the bioavailability of oral DES and stilphostrol in dogs, and the tissue distribution of DES versus stilphostrol in rats. The details of the methods used in that work, as well as two modifications of that method, are described here.

MATERIALS AND METHODS

Gas chromatographic–mass spectrometric assay for diethylstilbestrol

Chemicals and drugs. The DES was a gift from Eli Lilly (Indianapolis, IN, U.S.A.). The internal standard, dimethylstilbestrol (DMS), was obtained from the National Cancer Institute, Drug Development Branch (Bethesda, MD, U.S.A.). DMS was made up in methanol as a 1 mg/ml solution. This was diluted 1000-fold for spiking samples. Certain DES metabolites were kindly provided by Dr. M. Metzler. Citric acid and disodium phosphate were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Dichloromethane (glass-distilled) was purchased from MCB (Cincinnati, OH, U.S.A.). Sodium heparin was manufactured by Upjohn (Kalamazoo, MI, U.S.A.). Trifluoroacetic anhydride (TFAA) was purchased from Pierce (Rockford, IL, U.S.A.).

Preparation of plasma sample. After dosing with either DES or stilphostrol, whole blood was collected from dogs, rats or humans. The blood samples were placed in B.-D. Vacutainers (Becton-Dickenson, Rutherford, NJ, U.S.A.), which contained 0.07 ml of 15% tripotassium EDTA and to which we added 0.1 ml of 1 *M* L(+)-tartaric acid. The sample was immediately placed on ice. The whole blood was then centrifuged at 1500 *g* for 10 min and the plasma was removed. Except for certain human experiments, control plasma was also collected from each subject before dosing. The internal standard, DMS (50 ng), and 1 ml of 0.1 *M* citrate–0.062 *M* phosphate buffer (92:8, v/v, pH 5.8) were added to 1 ml of plasma. For each set of samples to be analyzed, standard curves were produced with DES-spiked control plasma (Fisher, Sera Chem) and processed under identical conditions used for the experimental samples. Methylene chloride (3 ml) was added to each buffered plasma sample. The tubes were capped and gently shaken in a horizontal position for 15 min. They were then centrifuged for 10 min at 700 *g*. The organic layer was transferred to 1-ml tapered-bottom reaction vials and evaporated to dryness under a gentle stream of nitrogen in a 60°C heating block. After drying, the samples were usually stored at −20°C pending the remainder of the analysis. Samples were next derivatized with 250 μl of dichloromethane–TFAA (3:1, v/v) for 20 min at room temperature. Finally, the dichloromethane–TFAA solution was evaporated at room temperature under a gentle stream of nitrogen and the samples were reconstituted with 30–40 μl of dichloromethane for analysis. The samples must be analyzed as soon as possible after derivatization because of decomposition after 12–14 h.

Preparation of tissue sample. Rats were given doses of DES by tail vein and sacrificed 5 h later. They were anesthetized with diethyl ether and exsanguinated from the juncture of the abdominal aorta and the iliac arteries.

The liver and prostate were removed, along with approximately 1 g of abdominal muscle. These samples were frozen until analysis. The prostate and a 0.2-g sample of the liver or muscle were individually homogenized using a Polytron (Brinkman, Westbury, NY, U.S.A.) in 4 ml of buffer. This homogenate was treated as the plasma samples described earlier. Standard curves for tissue samples were generated by spiking blank tissues from untreated animals.

Sample analysis. A Model 2700 gas chromatograph (Varian, Palo Alto, CA, U.S.A.) was directly coupled to a DuPont 21-491B mass spectrometer. Electron-impact ionization was used along with a four-channel multiple selected-ion detector. The chromatographic column was a narrow-bore fused-silica capillary, 25 m in length and coated with OV-101 (Hewlett-Packard, Palo Alto, CA, U.S.A.). A short section of the column was used as a transfer line to introduce the sample directly into the mass spectrometer ion source. The carrier gas was helium with a flow-rate of 2 ml/min. The gas chromatograph oven was temperature-programmed from 150°C to 225°C at 15°C/min. The injection port, transfer line, and mass spectrometer ion source temperatures were set at 250°C.

Of the derivatized sample 2 μl were loaded into a 10-μl syringe and fronted with 1 μl of dodecane to affect splitless injection. The 3 μl of liquid were injected over a 30-sec period followed by a 1-min wait before the injection port split valve was opened and the GC temperature programme started. This time is called zero for calculating retention times. After another 2 min to allow the solvent front to pass, a microcapillary T bypass valve (Precision Sampling, Baton Rouge, LA, U.S.A.) was opened to admit the helium stream into the mass spectrometer.

The mass spectra of the bistrifluoroacetyl derivatives of DES and DMS showed intense molecular ions at *m/z* 460 and 432, respectively. For routine analyses the multiple selected-ion detector was tuned to these molecular ions along with a confirmatory mass for DES (*m/z* 431). Because separation of the *cis-* (or *Z-*) and *trans-* (or *E-*) isomers of DES and DMS occurred, the peak heights of the appropriate isomers were summed for the ratio calculations. Because of the essentially constant coefficient of variation throughout the concentration range, linear regressions of calibration data were performed following a log—log transformation of the data.

Alkaline phosphatase assay for stilphostrol

Alkaline phosphatase (No. P 3877) was obtained from Sigma (St. Louis, MO, U.S.A.) and the other chemicals were obtained from Fisher and prepared to obtain the following concentration: 0.1 *M* Tris buffer (pH 8.5), 0.2 *M* glycine buffer (pH 8.8) and 0.05 *M* magnesium chloride. When plasma samples were collected for analysis, the tubes were pretreated with 10 mg EDTA and 0.1 ml of 1 *M* L(+)-tartaric acid for 5 ml whole blood. The former was used as an anticoagulant and an inhibitor of endogenous alkaline phosphatase and the latter inhibits acid phosphatase activity. Under these conditions there was negligible conversion of stilphostrol to DES. The alkaline phosphatase was prepared fresh daily at 2 mg/ml in the Tris buffer. Of this solution 5 ml were mixed with 2.0 ml of glycine buffer and 1.0 ml of magnesium chloride. Of the

pretreated plasma sample 1 ml was added and the mixture incubated at 35°C for 45 min. The internal standard, DMS, was added, and the sample was extracted and assayed for DES as previously described.

With this procedure linearity through a plasma stilphostrol concentration range of 5—5000 ng/ml was routinely obtained which was consistent with most of our biological samples.

High performance liquid chromatographic (HPLC) separation of stilphostrol and DES

For these experiments, an Altex 110A pump (Beckman, Berkeley, CA, U.S.A.) with a Rheodyne injector (Rheodyne, Corti, CA, U.S.A.) and a 100-μl sample loop was used. The separation was carried out on an Altex C_{18} reversed-phase column, 25 cm long, packed with 5-μm particles. An RP-18 5-μm guard column (Brownlee Labs., Santa Clara, CA, U.S.A.) was also used between the injector and the column. An LDC 1203 UV monitor (Milton Roy, Riviera Beach, FL, U.S.A.) detected the eluting compounds at 254 nm. The mobile phase was 0.004 *M* tetrabutylammonium hydroxide (Kodak, Rochester, NY, U.S.A.)—methanol (MCB, HPLC grade) (30:70, v/v). The flow-rate was 2 ml/min and the retention times for stilphostrol and DES were 1.8 and 5.2 min, respectively.

RESULTS AND DISCUSSION

Capillary GC—MS analysis of DES in plasma and tissues

The electron-impact mass spectrum of bis(trifluoroacetyl)diethylstilbestrol showed a molecular ion at *m/z* 460 which was the base peak, and the loss of an ethyl group producing another major ion at *m/z* 431. The internal standard, dimethylstilbestrol, also gave a molecular ion (*m/z* 432) as the base peak of its trifluoroacetylated derivative.

Using these molecular ions to detect each compound, and *m/z* 431 to confirm the presence of DES, linear standard curves could be generated over a wide concentration range. When conventional oral doses of DES (1—5 mg per day) were used, the resultant plasma concentrations averaged 2.4 ng/ml [5]. Calibration data between 0.3 and 5 ng/ml DES were summed over four separate days of analysis. The coefficient of variation was 25% and the regression coefficient (r) was 0.999. In experiments where intravenous DES or stilphostrol was given, the plasma DES concentrations were as high as 50 000 ng/ml. The calibration curve was linear throughout this concentration range.

Even though this is a highly sensitive method, its selectivity remained excellent when analyzing either plasma or tissues. Fig. 1 shows selected-ion recordings for plasma, muscle, liver, and prostate in untreated rats. In each case the *m/z* 460 tracing representing DES is free from interfering substances. The detection limit for DES in tissue (ng/g) was comparable to that in plasma (ng/ml).

The analysis of tissues from rats treated with DES before sacrifice are shown in Fig. 2. The indicated concentrations are those calculated from calibration data. There was no detectable DES in the muscle sample.

These chromatograms show that the relatively straightforward sample

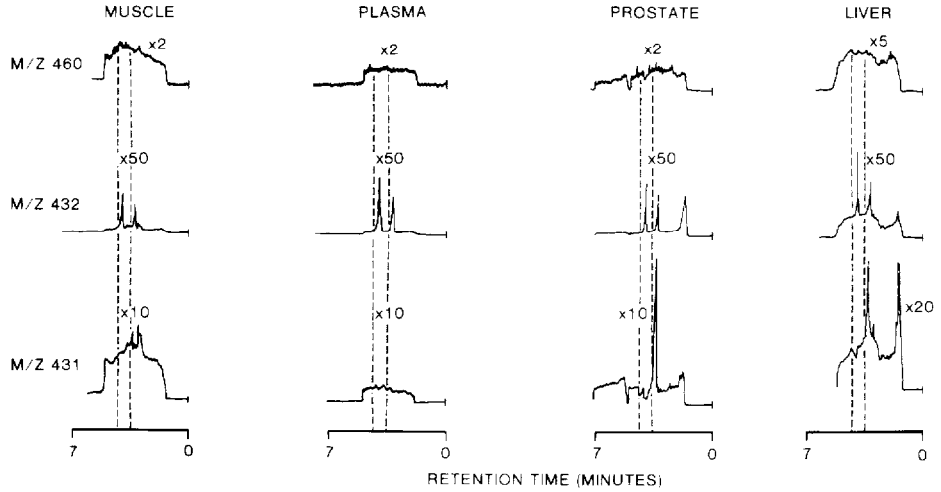

Fig. 1. GC—MS assay of diethylstilbestrol from plasma, muscle, liver, and prostate from an untreated rat. The top trace, *m/z* 460, quantifies DES, the middle trace, *m/z* 432, quantifies the internal standard, DMS, and the lower trace helps to confirm the identity of the DES peaks. The abrupt alterations in baseline are where the GC—MS isolation valve is opened or closed. The attenuation values are indicated for each trace. The vertical lines indicate the expected positions of the DES isomers.

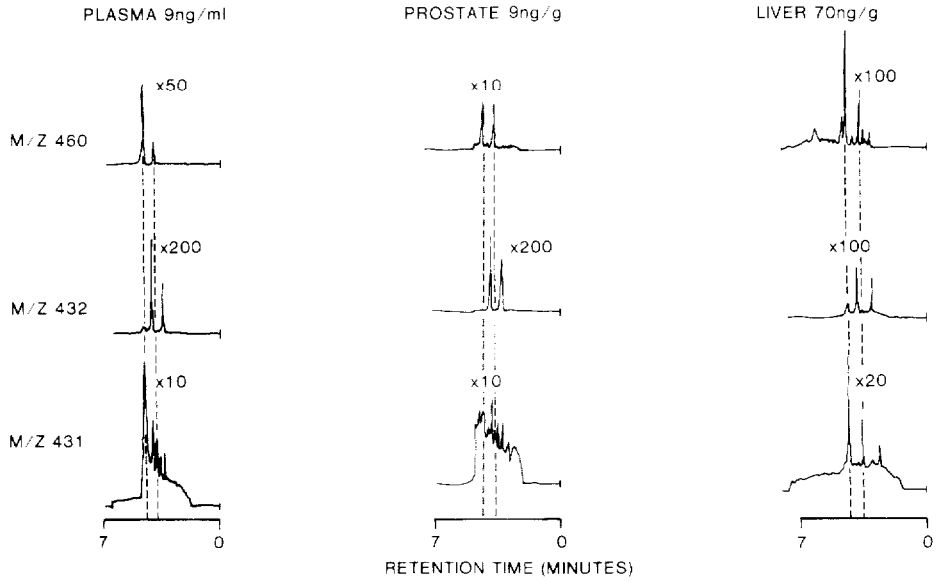

Fig. 2. Assay for DES from plasma and tissues from treated animals. The blood specimen was obtained 5 h after injecting a rat with 1.2 mg/kg DES. The liver was obtained 18 h after a similar DES dose. The prostate sample was taken 5 h after a 2 mg/kg dose of stilphostrol. The indicated concentration values are those calculated from calibration data. See the legend of Fig. 1 for other explanations.

processing which precedes the GC—MS assay produces a sufficiently clean sample to permit its use for its intended purpose, namely to assay for DES in pharmacokinetic studies. The clean blank plasma and tissue results indicate

that the other peaks seen in the *m/z* 460 channel for liver or in the *m/z* 431 channel for all the tissues shown in Fig. 2 are associated with the DES treatment. Because these metabolites have similar structures to DES (see below), higher chromatographic resolution might be preferable to more rigorous sample purification if this assay were to be used for regulatory purposes.

The metabolism of DES is complex with a variety of ring- and side-chain modifications [6]. Fig. 3 shows a number of these structures. We had authentic samples for several metabolites, but others were not available. Because the molecular ions were abundant in the mass spectra of all compounds examined, and the loss of the alkyl substituent on the olefinic stilbene carbons was also present, we searched for a number of metabolites in the plasma of a rat given a 20 mg/kg dose of stilphostrol intravenously and sacrificed 1 h later.

We did not attempt to quantify these metabolites. Our criteria for detection were that: the molecular ion had to be stronger than the $(M - 15)^+$ or the $(M - 29)^+$ ions except for metabolites such as 1-hydroxy-DES where the loss of a $CF_3COOC_2H_4$ $(M - 141)^+$ appeared to be predominant or 4′-methoxy-DES

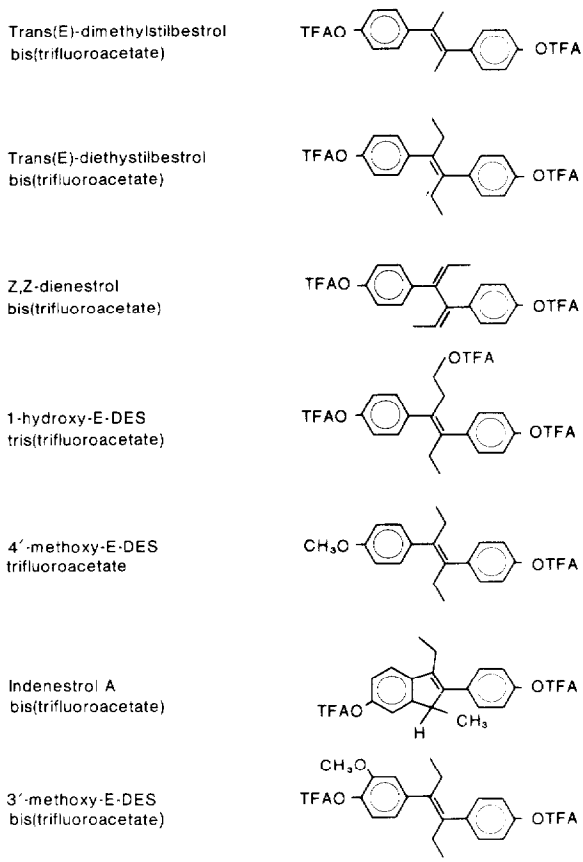

Fig. 3. Structures of diethylstilbestrol and related compounds as their trifluoroacetylated derivatives. The stereochemistry and positions of substituents are those presented in ref. 6 but are not necessarily representative of the specific compounds detected in the present work. TFA = trifluoroacetyl.

where loss of a methyl group predominated; that there were two peaks representing the *cis*- (or *Z*-) and *trans*- (or *E*-) configurations of each compound (except for indenestrol where no isomer is possible and dienestrol with three isomers); that these two peaks were in reasonable proportion to each other (generally the earlier eluting *cis* should be smaller than the later eluting *trans*); that the difference in the retention times of these two isomers be approximately 1 min; and that their overall retention times be consistent with their structures. Table I shows our data for the retention times of those compounds which met the preceding criteria. All were detected in one or more plasma samples from this rat.

TABLE I

RETENTION TIMES AND SELECTED MASSES FOR DIETHYLSTILBESTROL AND RELATED COMPOUNDS

Compound	Masses monitored*	Retention time (min)**
Dimethylstilbestrol	432	3.2, 4.0
Diethylstilbestrol	431, 460	3.6, 4.4
Dienestrol	458	3.6, 4.2
Hydroxy-DES***	431, 543, 572	3.6, 4.6
4'-Methoxy-DES***	349, 363, 378	3.8, 4.6
Indenestrol	458	4.6
Methoxy-DES***	461, 475, 490	4.8, 6.2

*The highest mass is the molecular ion of the fully trifluoroacetylated derivative.
**Multiple retention times refer to isomers of the same compound.
***Identity is speculative since no authentic samples were available.

Analysis of phosphorylated DES in plasma using alkaline phosphatase

To extend our analytical capabilities beyond DES itself, we adapted the method of Hare et al. [7] to the analysis of stilphostrol and DES monophosphate. The liberation of DES from stilphostrol by alkaline phosphatase incubation under the conditions described here was 98% complete. If the amount of alkaline phosphatase was halved, the conversion was 95% complete if the incubation time was doubled.

The overall assay for stilphostrol following alkaline phosphatase hydrolysis and analysis of released DES was less sensitive than the direct assay for native DES. The lowest reproducibly quantifiable concentration of stilphostrol was 1 ng/ml and the highest measured plasma stilphostrol concentration was 150 000 ng/ml from a dog given 250 mg stilphostrol intravenously.

Blood samples were taken at 1, 2, 4, 8 and 16 min from three rats which had received a 2.0 mg/kg dose of stilphostrol. These samples were subjected to the alkaline phosphatase assay to describe the behavior of the phosphorylated entities, stilphostrol combined with DES monophosphate. The 1-min concentration of the total phosphorylated species averaged 15 μg/ml and the directly measured DES concentration was 500 ng/ml. By 16 min the total phosphorylated species had declined to 870 ng/ml while the DES was just below 400 ng/ml. Thus, the phosphorylated species, predominantly stilphostrol, were very rapidly cleared from the plasma. This rapid clearance was probably a result of both hydrolysis in plasma and distribution into tissues.

The concentration versus time course of the phosphorylated species during this time period followed a biexponential decay, with half-lives of approximately 1 and 5 min.

Ion-paired reversed-phase HPLC separation of stilphostrol and DES

To increase the specificity of the assay for the different phosphorylated forms of DES, we attempted to separate stilphostrol and DES monophosphate from DES with HPLC. Each compound was collected and incubated with alkaline phosphatase. This was followed by a separate GC—MS analysis of each collected and processed specimen for DES. Because DES is a very lipophilic, nearly water-insoluble compound, and stilphostrol is a diphosphate, their chromatographic properties are different such that a suitable isocratic HPLC separation without ion pairing could not be obtained.

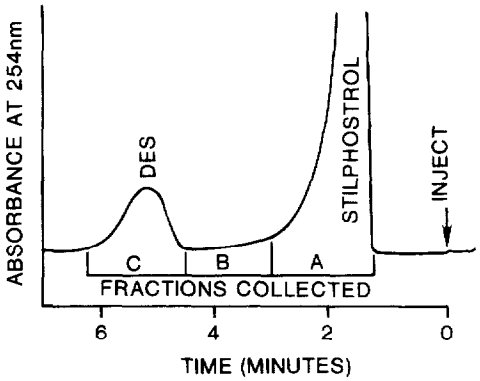

Fig. 4. Reversed-phase, ion-paired separation of stilphostrol and DES. Fractions for subsequent alkaline phosphatase hydrolysis were collected during the intervals marked. Band A contained stilphostrol, band C contained DES, and band B was expected to contain diethylstilbestrol monophosphate.

A chromatogram showing the retention of authentic stilphostrol and DES appears in Fig. 4. The vertical lines show the collected portions for each of the three assayed compounds. The positioning of DES monophosphate between stilphostrol and DES is speculative. When three dogs were given a 10-mg intravenous dose of stilphostrol, the DES which was liberated from the band marked B in Fig. 4 (presumably representing DES monophosphate) was somewhat less than 10% of that contained in the stilphostrol portion (band A). These data suggest that the hydrolysis of DES monophosphate to DES is much faster than the hydrolysis of stilphostrol to DES monophosphate. Thus, the concentration of the intermediate monophosphate is always low. The kinetics of band B did not resemble that for band A. Thus, whatever we measure in B is unlikely to be solely due to carry-over from band A. Clearly, more information is needed before declaring this a definitive method for analysis of DES monophosphate.

This capillary GC—MS method has proven to be a reliable procedure over a period of two years of continuous use. It was occasionally necessary to replace the injection port liner and the first few inches of the column when the chromatography of samples containing low DES concentrations appeared to decrease in efficiency.

The only other GC—MS assay described for studying the human pharmacokinetics of DES was that of Kemp et al. [4]. They used solvent extraction, followed by column chromatographic clean-up, which was then followed by a silylation reaction. Packed-column chromatography was used to separate each component whose molecular ions were detected using a high-resolution mass spectrometer. Their limits of quantitation were below 0.5 ng/ml from 10-ml plasma samples. We have been able to achieve this level of performance from 1-ml samples without complex purification procedures. The use of capillary columns rather than packed columns, and the use of the more selective acetylation procedure rather than silylation may explain why our method required less sample processing. We have carried out numerous studies of DES in man, dog, and rat, without observing any significant interferences.

We have also described an HPLC separation procedure for phosphorylated DES which precedes an enzymatic deconjugation step and the GC—MS assay. This technique has not been fully evaluated by a long period of use, but it may be more definitive than measuring the concentration of phosphorylated species by subtraction of a prehydrolysis value from a posthydrolysis value. The use of ion-paired, reversed-phase HPLC appears to have general applicability to drugs and their conjugates with hydrophilic acids, such as phosphates, sulfates, glucuronides, and peptides.

ACKNOWLEDGEMENTS

This work was supported by USPHS Grant CA 20753. The clinical collaboration of Dr. Harry C. Miller, Jr. is gratefully acknowledged.

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