Journal of Chromatography, 357 (1986) 416–422
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 489

Note

Coupled high-performance liquid chromatography–capillary gas chromatography as a replacement for gas chromatography–mass spectrometry in the determination of diethylstilbestrol in bovine urine

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(Received January 23rd, 1986)

In many instances it is desirable to replace coupled gas chromatography–mass spectrometry (GC–MS) by cheaper methods more suitable for the routine analyses of large numbers of samples, which implies first of all automation. GC–MS is mainly used for two reasons: first, if an analysis requires highly specific detection owing to presence of interfering peaks, *i.e.*, if the sample clean-up does not isolate the compound(s) of interest sufficiently well from the remainder of the sample; second, if the available GC detection techniques, *e.g.*, flame ionization detection (FID), do not provide sufficient sensitivity. Selected ion monitoring (SIM) in MS enhances the sensitivity compared with FID by at least one order of magnitude.

Coupled high-performance liquid chromatography–gas chromatography (HPLC–GC) involving standard GC detectors is attractive for replacing GC–MS, as the highly efficient sample clean-up by HPLC often readily allows the two requirements described to be fulfilled: first, less selective detectors are suitable because of fewer interfering components and second, a larger aliquot of sample can be injected owing to the reduced intensity of sample matrix effects (interfering peaks, "dirt" effects), which enables the detection limits to be lowered.

The determination of diethylstilbestrol (DES) in bovine urine (in many countries the use of DES for accelerating growth is illegal) is a typical example of applications where in many laboratories large numbers of analyses are carried out by GC–MS, *e.g.*, according to ref. 1. Screening methods that circumvent GC–MS are available for this particular analysis, such as radioimmunoassay (RIA)[2] or pure HPLC methods (*e.g.*, ref. 3). Nevertheless, the analysis of DES was selected for experiments with HPLC–GC as it was felt that this method would be highly competitive with the existing methods. Particular emphasis was placed on a comparison of the achievable sensitivity with that obtained in our laboratory using the same sample preparation followed by GC–MS, whereby none of the methods was pushed beyond the routinely readily available to the ultimately possible sensitivity.

EXPERIMENTAL

Sample preparation

A 10-ml volume of bovine urine was hydrolysed enzymatically with

0021-9673/86/$03.50

glucuronidase–sulphatase overnight at 40°C. The hydrolysed sample was loaded on to a Sep-Pak column, rinsed with 10 ml of 40% methanol and eluted with 3 ml of peroxide-free tetrahydrofuran (THF). The THF was evaporated and the residue was dissolved in diethyl ether and dried with anhydrous sodium sulphate. The dipentafluorobenzyl ether derivative was prepared according to ref. 4 using pentafluorobenzyl bromide. The sample was evaporated to dryness and the residue dissolved in 100 μl of cyclohexane–1% THF.

HPLC separation

A 100 × 3 mm I.D. glass column was used, packed with Spherisorb S-5-W. Cyclohexane–1% THF was used as the eluent at a flow-rate of 260 μl/min. The derivatized DES was detected at 230 nm.

HPLC–GC interface

The outlet of the HPLC detector was mounted in a four-port switching valve as described in ref. 5, allowing direct introduction of the eluent into the inlet of the retention gap. The internal volume of the connection between the HPLC detector cell and the switching point was 30 μl, causing the arrival of the detected solute material at the switching point to be delayed by 6 s.

GC system

A 15 m × 0.32 mm I.D. glass capillary coated with OV-73 of 0.25 μm film thickness was fused[6] with a 50 m × 0.32 mm I.D. retention gap of glass, silylated with hexamethyldisilazane. The carrier gas (hydrogen) inlet pressure was 1.6 atm. Sample introduction occurred at 80°C, followed by solvent evaporation at 100°C (for optimization of solvent evaporation, see ref. 7) and ballistic heating to 180°C. DES eluted at 270°C after programming the oven temperature at 4°C/min.

Detector by-passing

A system resembling that proposed by Schomburg[8] was used to prevent passage of the solvent vapour through the electron-capture detector. The detector (ECD 20, Carlo Erba) was modified to allow the make-up gas (nitrogen at 40 ml/min) to enter from the top of the detector and to flush the vapours eluted from the column exit away from the detector cell into the base of the detector block and through the line usually used as the inlet for the make-up gas to the atmosphere. This was achieved by feeding the make-up gas through a four-port valve (Valco) either into the detector base as usual (for detection of solutes) or into the top of the detector (by-passing solvent).

The system avoiding passage of the solvent vapour through the detector was constructed because of concern about contaminating the detector cell. Later, dozens of injections were carried out, each time directly introducing the vapour of about 300 μl of HPLC eluent into the detector. Detector performance was not affected noticeably. The question of whether large volumes of solvent vapour cause deterioration of electron-capture detectors is still open.

Procedure

The HPLC system was optimized to elute the DES fraction with an eluent

volume of 300 μl (carried out with pure standard). Then the sample was analysed on the HPLC system under relatively high attenuation of the detector to obtain a reasonable chromatogram of the sample matrix (for a typical sample as shown in Fig. 1). This sample was spiked with a fairly large amount of DES for determining the relevant fraction in the chromatogram. The DES fraction transferred into the GC system was cut according to the HPLC trace rather than trusting in the stability of absolute retention times (a problem primarily caused by instability of the eluent flow-rate).

The GC conditions were optimized in parallel, injecting DES on-column with a syringe (as the retention gap is mounted into the on-column injector also in coupled HPLC–GC, it is sufficient to take the transfer capillary out of the injector to allow normal on-column injection with a syringe). The transfer efficiency and the overall yields were tested by comparison of the peak areas obtained by directly injecting 1 ng of DES into the GC system, injecting the same amount of DES into the HPLC system followed by transfer into the GC system and adding it to the sample before working it up. Yields were around 95 and 70% respectively.

For introduction of the DES fraction into the GC system, the valve in the transfer line was switched 6 s after the appearance of the critical point in the HPLC trace. Transfer of the fraction took 70 s.

The HPLC column was reconditioned during the GC analysis (lasting *ca.* 45 min, including cooling of the oven at the end of the analysis), using 10% THF–cyclohexane. After about 30 injections of samples the column was cleaned using THF alone.

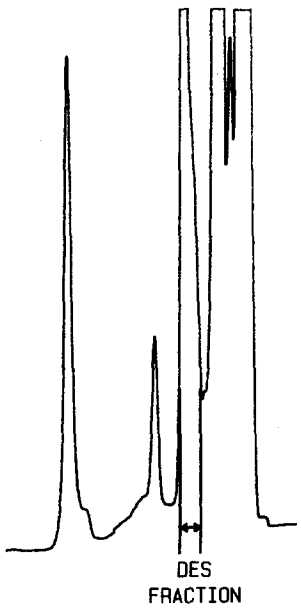

Fig. 1. HPLC trace of a typical sample after derivatization of DES to the dipentafluorobenzyl ether. UV detection at 230 nm. The transferred DES fraction almost corresponds to a peak in the HPLC trace but, of course, this peak does not represent DES.

RESULTS

Experiments involving FID

Initially, experiments were carried out using FID and preparing the heptafluorobutyro derivative of DES. These experiments provided interesting information on the sensitivity achieved with FID. However, decomposition of the derivative in the HPLC column after injection of a few samples caused severe problems, which prompted us to prepare another derivative (one of the not very common examples where a compound is perfectly stable in GC but not in HPLC!).

Fig. 2 shows the chromatogram of a sample free from DES obtained with FID. The position and height of a peak corresponding to 10 ppb* of DES, added to the same sample before the clean-up, was transferred from another chromatogram and is shown as a bar. The practical detection limit was *ca.* 3 ppb (1–4 ppb for other samples using the same criteria). This sensitivity was achieved injecting a 10-μl vol-

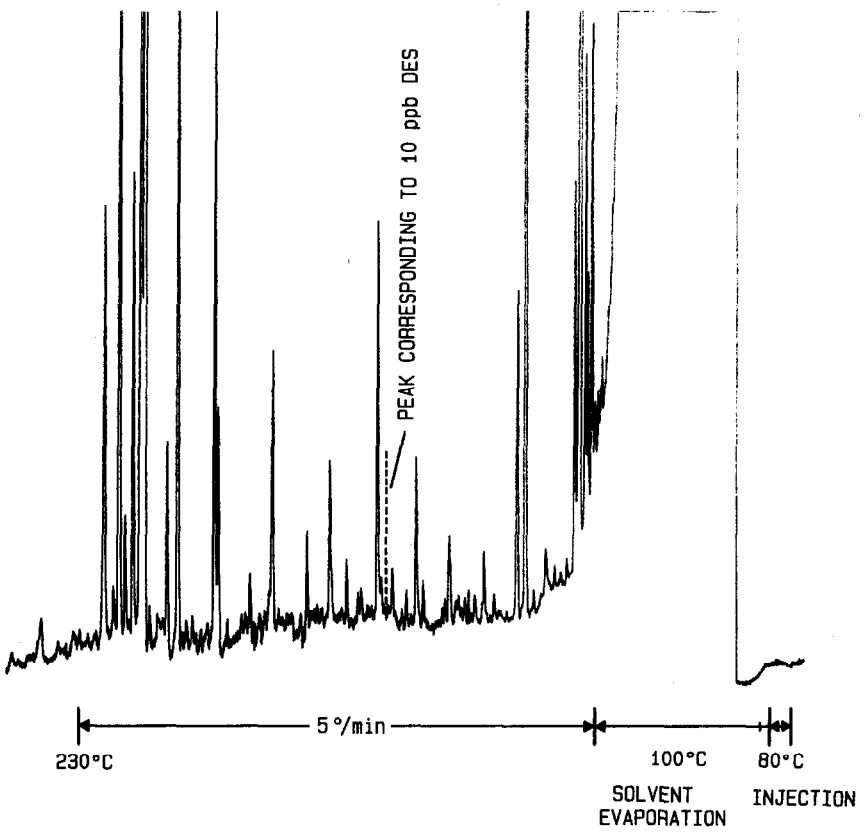

Fig. 2. FID chromatogram of the DES fraction obtained after formation of the heptafluorobutyro derivative of DES. The position and height of the (main) peak corresponding to 10 ppb of DES in urine are shown.

* Throughout this article the American billion (10^9) is meant.

ume from the 100-μl volume of prepared sample into the HPLC (a 3 ppb concentration corresponding to 3 ng of DES). This volume could easily be increased to 30–50 μl, but the reduction in the detection limit is small, and the smaller injection volume was preferred in order to reduce the load of sample by-products on the HPLC column to a minimum.

The level of sensitivity obtained with FID is similar to that routinely obtained by GC–MS using the same sample preparation except that the bis(trimethylsilyl) derivative of DES is prepared. Of course, the amounts of sample material injected were different (as the absolute sensitivity of MS is higher than that of FID). For HPLC–GC an extract corresponding to 1 ml of urine was injected (10% of the extract from a 10-ml volume of sample). For GC–MS only the extract of about 200 μl of

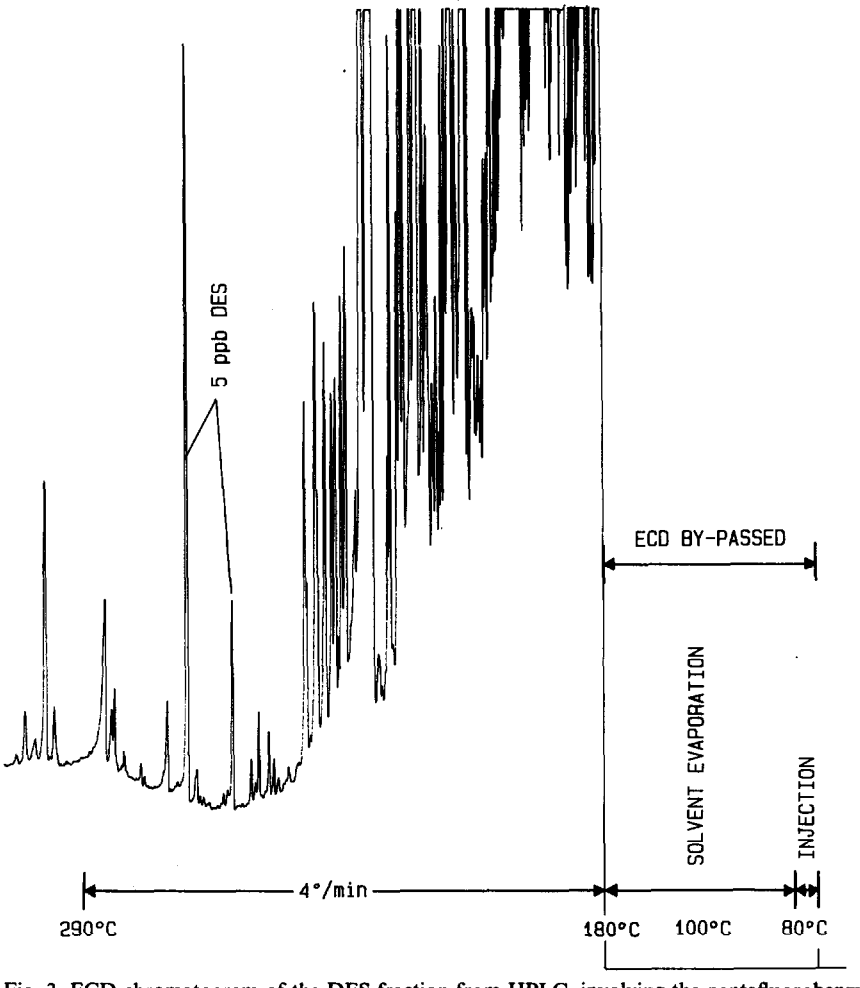

Fig. 3. ECD chromatogram of the DES fraction from HPLC, involving the pentafluorobenzyl ether derivative of the two isomers of DES. Sample spiked with 5 ppb of DES before sample preparation. During passage of the solvent vapour through the column, the column eluent was driven away from the detector cell through the line usually used for feeding the make-up gas into the detector.

urine could be injected (2 μl from 100 μl of extract). Injection of larger aliquots of sample into the GC–MS system increased the absolute peak sizes, but did not improve the detection limits owing to the simultaneously amplified sample matrix.

Experiments involving electron-capture detection (ECD)

The experiments involving ECD were carried out using the pentafluorobenzyl ether derivative of DES, as described under Experimental. The chromatogram of a typical sample spiked with 5 ppb of DES is shown in Fig. 3. The practical detection limits varied between about 0.1 and 0.3 ppb, depending on the urine analysed. There is even some room for improvement if a higher sensitivity is desirable: the injection volume (10 μl) could be increased several-fold. The detector attenuation was high, although the detector showed only about a fifth of the sensitivity of a new instrument.

DISCUSSION

HPLC–GC is an attractive method for routine analyses of trace components in complex samples as it substantially decreases the detection limits through a far more efficient isolation of the fraction of interest. It improves the sensitivity achievable primarily by permitting analyses of larger aliquots of sample (allowing the injection of larger proportions, *e.g.*, of a sample extract).

The analysis of DES by HPLC–GC–FID provided a similar sensitivity to GC–MS. This might turn out to be a generally valid rule of thumb for all kinds of samples where the sample matrix determines the practical detection limits and for which no clean-up has been carried out involving pre-separation steps of an efficiency comparable to that of HPLC.

Using ECD, the sensitivity of the HPLC–GC method exceeded that obtained by GC–MS by at least a factor of ten. At a 0.1 ppb level of DES in urine an amount of 0.1–0.2 ng of solute reached the detector, which is far from being at the limit of sensitivity of this detector.

Of course, coupled HPLC–GC–MS would be the method of choice regarding sensitivity and specificity of detection. The sensitivity obtained by HPLC–GC–MS would correspond approximately to that of HPLC–GC–ECD, but the information obtained about the solute represented by the peak at the correct retention time is more specific when detected by MS.

Another potential aspect of HPLC–GC did not materialize during our work, *viz.*, automation of the procedure. It should be easy to build a system that allows relatively dirty samples to be loaded into an HPLC autosampler and to obtain GC results automatically. Automation of the transfer step requires a power valve and a timer and has been realized by Cortes *et al.*[9].

The work described here was carried out using a long retention gap according to the concept of negligible solvent evaporation during sample introduction[10]. Simultaneously we worked on a method of introducing the HPLC eluent through a sample loop, involving concurrent solvent evaporation[11]. This method is restricted to solutes eluted at elevated column temperatures (above about 150°C), but is very convenient and allows the introduction of considerably larger eluent volumes.

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