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DETERMINATION OF KOVÁTS RETENTION INDICES WITH A CAPILLARY COLUMN AND ELECTRON-CAPTURE DETECTION: APPLICATION TO THE ASSAY OF THE ENZYMATIC CONVERSION OF 3,4-EPOXY-1-BUTENE INTO DIEPOXYBUTANE

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SUMMARY

A method is described that allows for alternate operation of a "pin and cup"-type electron-capture detector (ECD) in the normal ECD mode and in the metastable argon ionization mode. By such means, marker *n*-alkanes can be run without a change of the gas or temperature parameters, and measurements can be made of the Kováts retention indices of unknown compounds that are detectable in trace amounts by ECD only. As an application, tests are reported which support the probable enzymatic conversion of 3,4-epoxy-1-butene into diepoxybutane during incubation of the former with rat liver microsomes.

INTRODUCTION

Previous studies by our group[1,2] have shown that simple electron capture detector (ECD) chromatographs, equipped with a solid injector of the moving-needle type and a wall-coated open-tubular (WCOT) column are convenient for certain enzyme kinetic studies involving a small amount of substrate and a low yield of metabolite detectable after simplified clean-up and derivatization.

Actually, the determination of Kováts retention indices[3] would greatly facilitate the interlaboratory comparisons of the results; the dependence of the Kováts retention index on the column polarity can give useful structural information as it relates to solvent–solute interaction during gas chromatography (GC)[4].

Recently, Grob *et al.*[5] utilized Kováts retention indices to show that, during the deactivation process of the glass of WCOT columns, over-silylation may falsify the retention indices due to extra retention of *n*-alkanes by support sites.

Up to now, the determination of Kováts retention indices with a single-channel ECD chromatograph has been difficult because ECD systems are optimized to give virtually no signal for small amounts of the marker *n*-alkanes.

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In this paper we report on the feasibility of occasionally running marker amounts of long-chain *n*-alkanes on such ECD systems.

"Pin and cup" ECD cells resemble the diode chamber previously described by Lovelock[6] for use as a general-purpose GC detector in the argon ionization mode; the cell is swept with argon without a quenching gas and the d.c. field strength is increased until the kinetic energy of free electrons is high enough to excite a sufficient proportion of sweeping gas to the metastable state without a corresponding increase in the concentration of ions[6]. The cell then detects at high sensitivity a number of organic molecules with ionization potentials below 11.6 eV.

We operated one such cell in the ECD mode with pure argon and the response was regular at low pulsed current values. In addition, it was simple quickly to switch over the metastable mode and obtain signals for marker *n*-alkanes. The method provided access to Kováts retention indices within the limits of precision inherent in run-to-run replication of retention times.

The experiments were carried out in the context of investigations of the enzymatic conversion of 3,4-epoxy-1-butene into diepoxybutane, catalysed by rat liver microsomes. A summary of assay method and of the results obtained is therefore also included.

EXPERIMENTAL

Gas chromatograph

The instrument was a Pye 104 isothermal oven equipped with a Chrompack 9000 solid injector for capillary columns, a 40 m × 0.5 mm I.D. WCOT column (3.6 μm SE-30 on polyethylene glycol-deactivated soda-glass; RSL, St. Martens Latem, Belgium), and a Pye Model 691649 electron-capture detector (10-mCi nickel-63). The cell had been previously in use and showed a slight but tolerable degree of contamination. Argon (L'Air Liquide, Liège, Belgium) of nominal purity 99.996% (residual nitrogen about 30 ppm) was used as the carrier gas after passage across a Chrompack charcoal filter[1] (column inlet pressure 30 cmHg, injector scavenging flow-rate 20 ml/min). Unfiltered argon was used for detector make-up at a rate of 50 ml/min. One-microlitre samples in *n*-hexane were applied to the plunger tip with the loading port open to the atmosphere[1].

Electron-capture detection

For the ECD mode, the detector was connected to a Pye Unicam Model 795022 pulsed constant-current amplifier. With argon without a quench gas, the runaway current was 1.3 nA.

Argon ionization detection for the n-alkanes

For operation at high field strength in the metastable mode, the detector coaxial cable was connected to the positive high-voltage (HV) terminal of a Pye Model 12309 HV/amplifier unit. This unit was originally developed for use with earthed-cathode argon detectors. It features a floating HV d.c. supply (up to 2000 V) and a linear ionization-current amplifier using semielectrometer tubes. When the unit was connected to the ECD cell at 150° with 50 ml/min of make-up argon, the detector sparked at about 1000 V; the value was reduced to 800 eV for detection. It is possible that the effective detector field strength was still lower, owing to the drop

across a built-in linearizing resistor in series (3000 MΩ). The signal was recorded with the amplifier at unity voltage gain. No attempt was made to calculate the standing ionization current. The baseline was fairly noisy; the apparent cause was insulation leakage at the aged HV unit rather than a detector effect.

For quick passage over the ECD mode, the detector lead was first earthed in order to remove static HV charges.

Chemicals

n-Alkanes were gifts from Labofina Laboratories (Brussels, Belgium). Pentafluorobenzoylphenoxyethanol was a gift from Dr. B. Rollmann. 3,4-Epoxy-1-butene, *meso*-erythritol and styrene glycol were obtained from Aldrich Europe (Beerse, Belgium); DL-1,2:3,4-diepoxybutane from Fluka (Buchs, Switzerland); DL-1,4-dichloro-2,3-butanediol from Eastman-Kodak (Rochester, N.Y., U.S.A.); pentafluorobenzoyl chloride from Macherey, Nagel & Co. (Düren, G.F.R.); and nanograde *n*-hexane from Mallinckrodt (St. Louis, Mo., U.S.A.). Other chemicals and solvents were of analytical-reagent grade.

Determination of corrected retention times and Kováts retention indices

Observance of a precise timing scheme, for manipulation of the injector over successive runs, was necessary. Indeed, with the "open" method of sample application[1], the column flow must be interrupted. Because the injector of relatively large volume is fed from a flow restrictor, an appreciable time elapses before pressure re-equilibration is attained after closure of the injection port. It was nevertheless possible, using the dropping of the injector needle as the zero mark, to reproduce to ± 1–2 sec retention times of the order of 15 min.

As another result of open injection on the WCOT column, the time elapsed between closure of the injection port and emergence of a negative air peak on the argon ionization chromatograms (Fig. 1), could not serve as a measure of column holdup (t_M). In addition, the physical air peak was inappropriate for measurements of the corrected retention times (t_R) of *n*-alkanes.

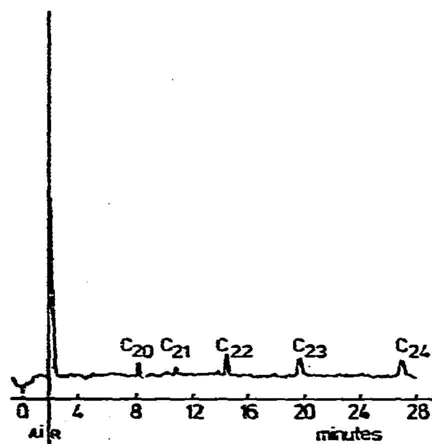

Fig. 1. Argon ionization chromatogram of *n*-alkanes (about 50 ng for C_{22}) at 209° using the ECD cell. Detector temperature 255°. Column and conditions as in the text.

It was therefore necessary first to compute the "true air peak" time from the retention distances between several adjacent *n*-alkanes. Injection of about 50 ng each of the members from C_{20} to C_{24} yielded usable peaks (Fig. 1), in spite of a large dilution of the column effluent by make-up argon. The method of computing t_R values was a modification[7] of the simplified method of Peterson and Hirsch[8] proposed by Ettre[9].

Unknown compounds were run in the ECD mode by using the same volume of *n*-hexane solvent and the same injection timing. Unknown compounds of interest in this work eluted between the C_{21} and C_{22} *n*-alkanes and their retention indices (i_x) were calculated according to Kováts[3].

APPLICATION: INVESTIGATIONS ON THE CONVERSION OF 3,4-EPOXY-1-BUTENE INTO DIEPOXYBUTANE

In the present study we investigated the possibility for the mixed-function oxidase system of rat liver microsomes to enzymatically convert 3,4-epoxy-1-butene (I) into butadiene diepoxide (II). It is known[10] that II in aqueous medium is hydrolysed to erythritol (III), which upon treatment with hydrochloric acid yields 1,4-dichloro-2,3-butanediol (IV).

Treatment of II with hydrochloric acid, derivatization and GC with ECD

One microgram of DL-1,2:3,4-diepoxybutane was allowed to hydrolyse slowly in water (1 ml). A 1-ml volume of 2 *N* hydrochloric acid was added, followed, after 10 min at room temperature, by 0.2 μg of styrene glycol in aqueous solution (internal standard). The mixture was stirred and extracted with ethyl acetate (2 ml). The ethyl acetate extract was evaporated to dryness under nitrogen and the residue was taken up in toluene (0.4 ml). Pentafluorobenzoyl chloride (4 ml) and pyridine (2 μl) were added and the mixture was kept sealed for 30 min at 60°. The cooled mixture was flushed to dryness with nitrogen and the residue was taken up in *n*-hexane (2 ml). The *n*-hexane phase was washed with 90% aqueous methanol (1 ml) and 1 μl of the *n*-hexane supernatant was chromatographed in the ECD mode (ionization current 0.1 nA). The resulting chromatogram is shown in Fig. 2. Under similar conditions, 10 pg of pentafluorobenzoylphenoxyethanol yielded a full-scale response at attenuation ×16.

Derivatization of IV

One microgram of DL-1,4-dichloro-2,3-butanediol in toluene (0.4 ml) was derivatized as above. The ECD chromatogram showed a single peak with the retention of A in Fig. 2. The Kováts retention index of peak A (2150 at 209°; 2155 at 239°) was judged to be independent of temperature owing to the error inherent in the present method of determination.

Treatment of meso-erythritol

meso-Erythritol (1 μg) was treated with 2 *N* hydrochloric acid (1 ml) for 10 min at room temperature. The subsequent steps of ethyl acetate extraction, derivatization and GC were carried out as above for II. The ECD record showed a major peak at the position of A and a small proportion of peak B (Fig. 2). The Kováts

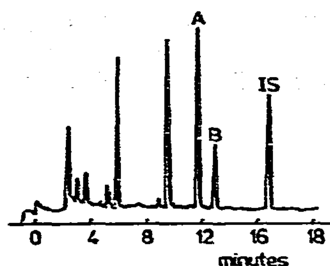

Fig. 2. ECD chromatogram of the product after hydrolysis of DL-1,2:3,4-diepoxybutane, treatment with HCl and derivatization with pentafluorobenzoyl chloride. A and B indicate peaks that are recurrent in experiments reported in the text. IS indicates the derivative from styrene glycol (internal standard). Temperatures: column, 222°; detector, 272°. Other conditions as in the text. Attenuation × 512.

retention indices for peak B were 2170 at 209° and 2190 at 239°, suggesting a slight positive temperature dependence.

Incubation of 3,4-epoxy-1-butene with rat liver microsomes

Microsomes from male Wistar rats were prepared according to the method of Amar-Costesec *et al.*[11]. The incubation mixture (2 ml) contained a Tris-hydrochloric acid buffer (50 m*M*, pH 7.8) and an NADPH-generating system. After pre-incubation for 10 min at 37°, microsomes, in an amount corresponding to 0.1–0.7 mg of protein per millilitre of incubation mixture, were added together with 25 μl of a 0.9 *M* solution of 3,4-epoxy-1-butene in acetonitrile. The incubation reaction was stopped after 10 min by addition of 2 *N* hydrochloric acid (1 ml). After 10 min at room temperature, 0.7 μg of styrene glycol in aqueous solution (internal standard) was added. The mix-

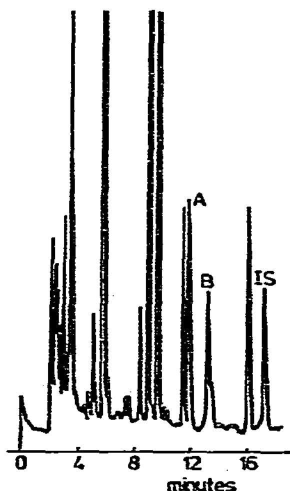

Fig. 3. ECD chromatogram of the *n*-hexane-extractable product after incubation of 3,4-epoxy-1-butene with rat liver microsomes, treatment with HCl and derivatization with pentafluorobenzoyl chloride. Attenuation × 32. Temperatures, peak notations and conditions as in Fig. 2.

ture was stirred and centrifuged at 3000 g. The supernatant was extracted with ethyl acetate (2 ml) and the ethyl acetate extract treated for derivatization and GC as above. Fig. 3 shows a typical ECD chromatogram where peaks A and B are recognizable with a peak-height ratio (A:B) of about 1.5:1.

DISCUSSION

Metabolism of butadiene

We recently demonstrated the capacity of the microsomal mixed-function oxidase system to convert butadiene into 3,4-epoxy-1-butene[12]. The present results deserve further identification work but already suggest a capacity of the same system to convert 3,4-epoxy-1-butene into diepoxybutane. Therefore, a net conversion of butadiene into diepoxybutane, by cellular systems, may be hypothesized. In several experimental models, diepoxybutane isomers manifested toxic, carcinogenic or mutagenic properties[13]; the problem might be raised of a potential hazard during human occupational exposure to butadiene.

Kováts retention indices

The adequate reporting of GC data constitutes one aspect of scientific communication. Unfortunately, the WCOT columns used for ECD are usually pre-tested with non-polar mixtures and FID, and there is as yet no general consensus as to reference compounds, preferably members of homologous series, that would be detectable at low levels with both ECD and FID and that could simultaneously provide the basis of a general indexing system. The present method of locating *n*-alkanes, using the ECD cell itself, allows for the use of a time-proven notation system and it avoids the sophistication of dual-detector instruments.

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