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ISOTHERMAL GAS CHROMATOGRAPHY WITH WALL-COATED GLASS CAPILLARY COLUMNS, ELECTRON-CAPTURE DETECTION AND A SOLID INJECTOR

II*. APPLICATION TO THE ASSAY OF 2-FLUORENYLACETAMIDE N-HYDROXYLASE ACTIVITY IN A RAT-LIVER MICROSOMAL SYSTEM*

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SUMMARY

The presumed superiority of wall-coated (WCOT) glass capillary columns with electron-capture detection (ECD) over packed columns and ECD was tested for the assay of N-hydroxy-2-fluorenylacetamide after a two-step derivatization into N-chloro-2-fluorenyltrifluoroacetamide or N-chloro-2-fluorenylmonochloroacetamide. Co-derivatization of N-hydroxy-4-biphenylacetamide yielded the internal standard. A commercial glass solid injector, short WCOT columns coated with a film of medium thickness of a non-polar phase and a macro-ECD cell were used isothermally with helium as the carrier gas. Repetitive analyses were possible with both derivatives at a speed unattainable with packed columns.

INTRODUCTION

A recent report from this laboratory[1] has described a rapid, specific and sensitive assay of N-hydroxy-2-fluorenylacetamide (N-OH-2-FAA) after a two-step transformation into N-chloro-2-fluorenyltrifluoroacetamide. The latter derivative could be quantitated by gas–liquid chromatography (GLC) on packed columns (3% OV-1) with electron-capture detection (ECD). When derivatization was carried out in the presence of a known amount of N-hydroxy-4-biphenylacetamide (N-OH-4-AABP) as the internal standard, the assay was suitable for the kinetic analysis of rat-liver microsomal 2-fluorenylacetamide N-hydroxylase. The study was part of a programme of investigations on the N-hydroxylation of arylamides, known[2] to represent an initial step in chemical carcinogenesis.

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A logical development of the assay of N-OH-2-FAA resided in the use of a wall-coated (WCOT) glass capillary column–ECD system, presumed to be more versatile for testing different electron-capturing acyl groups in the derivatization sequence. Among others, the monochloroacyl group could enhance the ECD response and produce a useful peak shift, thus reinforcing existing criteria of peak identity. A short capillary column was a practical necessity, as chloroacetates migrate slowly on packed columns at moderate temperatures.

When the project was started in 1977, it was not obvious that a WCOT column–ECD system could ensure continuing performance under conditions of rapid isothermal analysis at a high repetitive rate using crude derivatized extracts. Presumably, a rugged version of the all-glass solid injector would combine the advantages of splitless application of convenient sample sizes and accessibility to operators already familiar with packed column GLC.

The GLC system included a glass solid injector, WCOT columns coated with a medium-thickness film of a non-polar phase and a commercial "macro" ECD cell. The suppression of artefact peaks, arising from the solid injector, deserved special attention; conditions for stable operation were described in Part 1[3].

This paper describes further developments in the assay of N-OH-2-FAA using capillary column GLC–ECD. An improved preparation of the trifluoroacetate derivatives, a preparation of the monochloroacetates and their identification by mass spectrometry, and quantitative capillary column GLC of these derivatives are reported.

EXPERIMENTAL

Incubation of the rat hepatic microsomal system

Microsomes from male Wistar rats were prepared according to the method of Amar-Costesec *et al.*[4]. For evaluation of the N-hydroxylase activity, the incubation mixture (3 ml) contained a phosphate buffer (20 m*M*, pH 7.6) and an NADPH-generating system. After pre-incubation at 37°, microsomes corresponding to 0.4–0.8 mg wet weight of liver and N-2-fluorenylacetamide in methanol (50 μl; final concentration 0.25–5 μM) were added. The reaction was stopped after 10–15 min by addition of 0.27 *M* calcium chloride solution (50 μl) followed by heating for 10 min at 100°. After cooling a known amount (50–100 ng) of N-OH-4-AABP was added as an internal standard.

Derivatization and extraction for GLC–ECD

The previous mixture was centrifuged for 10 min at 2000 *g*. To the decanted supernatant, concentrated hydrochloric acid (0.25 ml) was added; the mixture was heated for 90 min at 85°. After cooling, extraction was carried out with cyclohexane (three 2-ml volumes). To the cyclohexane phase (6 ml) either trifluoroacetic anhydride (30 μl) or monochloroacetic anhydride (50 μl of a 0.5% solution in toluene) was added. After 2 min the solution was evaporated under nitrogen in a heating block at 60° and the residue was re-dissolved in cyclohexane (0.5 ml). For GLC of the trifluoracetates, aliquots of 1–2 μl of the extract were directly applied to the chromatograph. In the case of monochloroacetates, the extract was stirred with 1 *M* sodium hydroxide (0.2 ml) and the upper cyclohexane layer was used for GLC after centrifugation for 2 min at 2000 *g*.

Capillary column GLC with electron-capture detection

The instrument was a Pye 104 isothermal oven equipped with a Chrompack 9000 solid injector for capillary columns, a 5–6.5 m × 0.5 mm I.D. WCOT column and a Pye Unicam Model 795012 electron-capture detector. Helium (inlet pressure 15–30 cmHg; injector scavenging flow-rate 20–30 ml/min) was used as the carrier gas and argon–methane (95:5) for detector make-up (50 ml/min). A charcoal filter (Chrompack) was used in the carrier gas line and the samples were applied with the loading port open to the atmosphere. Further details were given in Part 1[3].

N-Chloro-N-monochloroacetates were analysed at 200–220° on Pyrex WCOT columns (gifts from Dr. Johan Bouche). The liquid phase was a film of SE-52 (thickness about 0.3 μm) coated without prior glass deactivation. N-Chloro-N-trifluoroacetates were run at 170–200° on both pyrex and soft-glass WCOT columns coated with SE-52 or SE-30. In one instance, a Pyrex WCOT used for the monochloroacetates was regenerated by using the deactivation method of Franken *et al.*[5], which involves exposure to the vapour bleeding from heated polyethylene glycol.

Identification of the derivatives by mass spectrometry

Mass spectrometric analyses were effected with an LKB 9000 instrument. N-Chloro-4-biphenylmonochloroacetamide was injected into the gas chromatograph with a flash heater temperature of 270°, a helium carrier gas flow-rate of 30 ml/min and an oven temperature of 250°. The column was a standard LKB coiled gas chromatographic column, 2.2 m × 5 mm O.D., packed with 1% OV-1 on 60–80-mesh Chromosorb W. N-Chloro-2-fluorenylmonochloroacetamide was introduced directly into the mass spectrometer. Mass spectra were recorded at 70 eV with an accelerating voltage of 3500 V; the trap current was 60 μA and the ion source temperature 270°.

RESULTS

Derivatization procedure

Mass spectra of the intermediate products in the two-step derivatization sequence (N-chloro-2-fluorenylamine and N-chloro-4-biphenylamine) and of N-chloro-2-fluorenyltrifluoroacetamide and N-chloro-4-biphenyltrifluoroacetamide were presented in a previous paper[1]. Mass spectra of the chloroderivatives are illustrated in Fig. 1 here. Losses of a chlorine atom and a O=C=CH–Cl molecule are observed for the two derivatives.

Quantitative GLC with ECD

In initial experiments the individual trifluoroacetates were used and each capillary chromatogram was duplicated on a proved packed column (3% OV-1) with ECD. At least once a day, the continuing performance was checked with organochlorine pesticide samples[3]. There was evidence that the assayed trifluoroacetates survived on both the Pyrex and soft-glass WCOT columns used. All of the analyses were carried out with open sampling and a charcoal filter placed in the carrier gas line, and there was no evidence of ghost peaks due to the injector cycle. Running large batches of samples in rapid sequence (3-min analysis time) was pos-

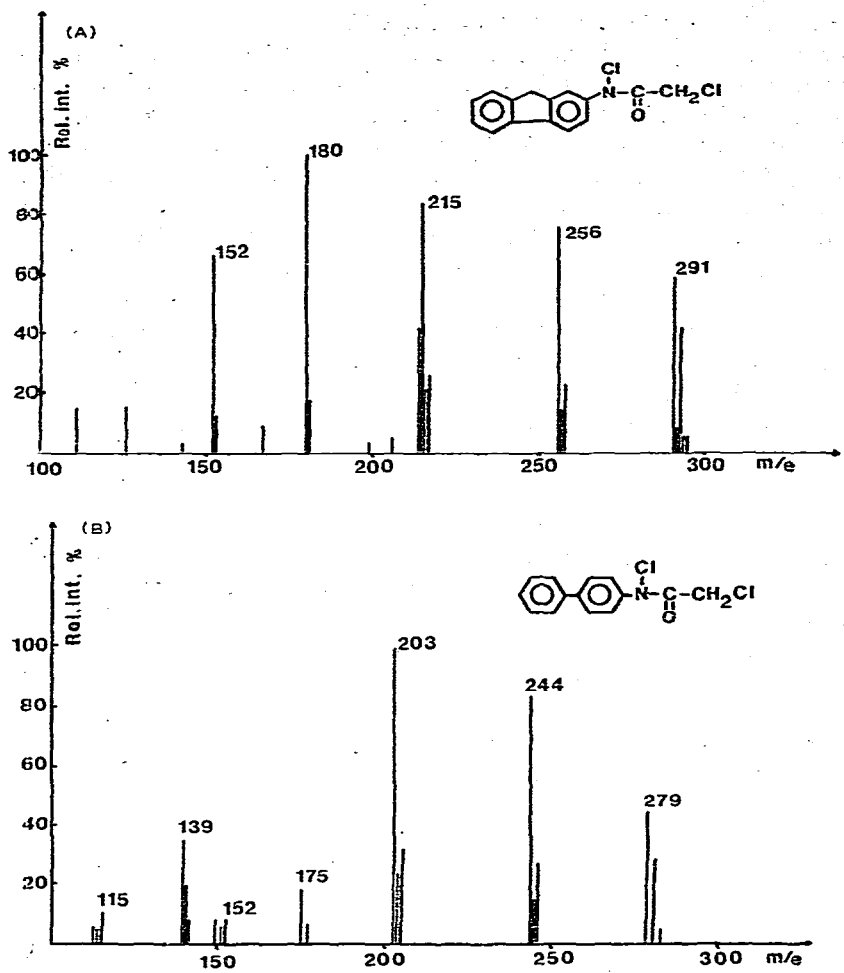

Fig. 1. Mass spectra of (A) N-chloro-2-fluorenylmonochloroacetamide and (B) N-chloro-4-biphenylmonochloroacetamide.

sible isothermally. Coating of the inlet surfaces by deposits was observed, but this did not affect the continuing performance.

The usable range of linear responses (based on peak-height ratios) was significantly improved in comparison with packed column calibrations. The working detection limit was calculated after addition of trifluoroacetates obtained on a preparative scale[1]. An amount of 20 pg (expressed as N-OH-2-FAA) could be detected with the capillary system, compared with 50 pg on packed columns. This was not due to exploitation of a greater inherent sensitivity (the attenuation was 4 or $\times 128$), but to better discrimination of the chemical background with the extracts.

A routine chromatogram of the trifluoroacetates is shown in Fig. 2A (6.5-m SE-52 column), obtained at a column temperature of 170°. At such a low tem-

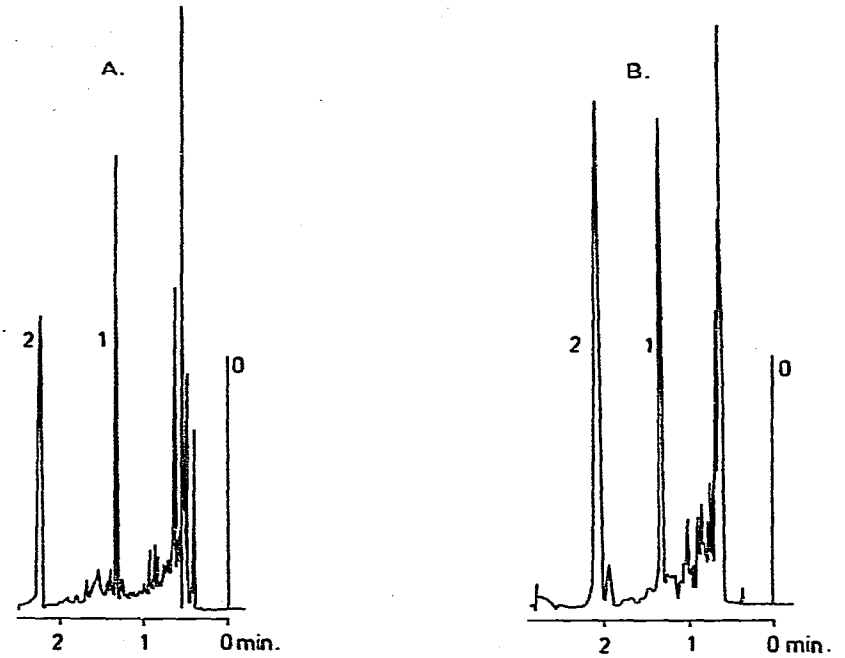

Fig. 2. Capillary column chromatograms of components in the N-hydroxylase assay as extracted from the incunation mixture and derivatized by two methods for EDC. (A) N-Chloro-N-trifluoroacetates; (B) N-chloro-N-monochloroacetates. Peaks: (parent compounds): 0 = injection; 1 = N-hydroxy-4-biphenylacetamide (internal standard); 2 = N-hydroxy-2-fluorenylacetamide. Conditions: 6.5-m SE-52 column. Helium carrier gas (30 cmHg inlet pressure); open sampling method: injector purge 25 ml/min. ECD make-up gas, argon–methane (55 ml/min). Attenuation, ×64. Column temperatures: (A) 170°; (B) 200°.

perature the retention time of the N-OH-2-FAA derivative was halved in comparison with the analysis on 6-ft columns packed with 3% OV-1 operated at 200°. Most impressive was the superior readability of the records from the capillary system, showing a considerable reduction in the tailing of the "solvent" front.

Fig. 2B shows a chromatogram of the monochloroacetates derived from a similar extract, on the same column at 200°. Four hundred analyses over 6 weeks showed a uniform performance. After this period the column, for an unknown reason, abruptly degraded, although the performance was conserved for the test pesticides. We cannot rule out that the change in column properties might correspond to the injection of impure samples. Preliminary results indicated that the degraded column could recover its initial performance for the monochloroacetates when the gas-phase deactivation method of Franken *et al.*[5] was applied.

Fig. 3 shows a calibration graph for the monochloroacetates. The assay conditions were as described under Experimental. The graph shows good linearity for N-OH-2-AAF concentrations ranging from 2 to 200 ng/ml. A comparison with results from a packed column was not possible using the monochloroacetates, as the 3% OV-1 columns available in this study failed to yield a response with these derivatives.

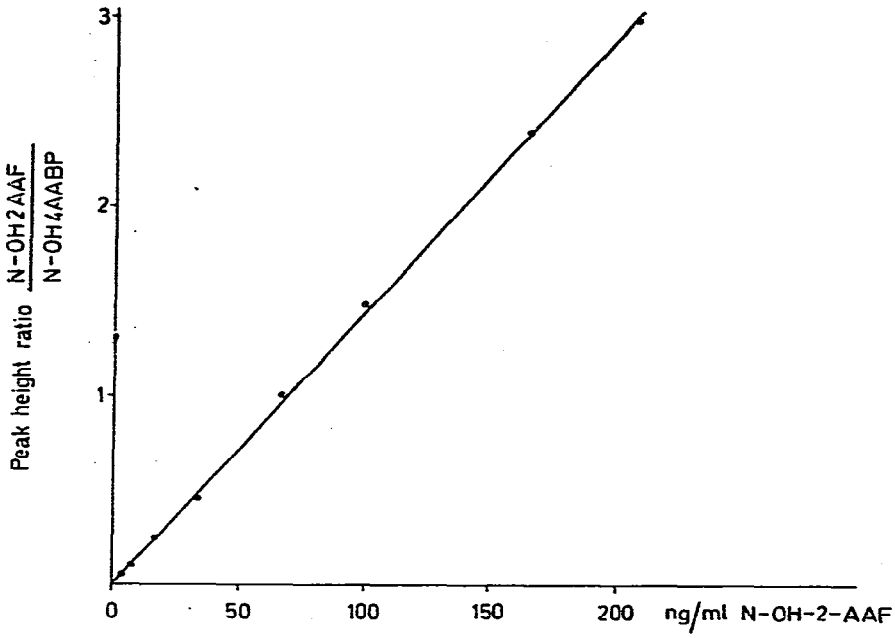

Fig. 3. Graph showing the linearity of capillary column GLC peak-height ratios (N-OH-2-AAF/N-OH-4-AABP, measured as the N-chloro-N-monochloroacetates) *versus* amounts of underivatized N-OH-2-AAF added to a simulated incubation mixture. GLC conditions as in Fig. 2B.

Instrumental performance

Over 7 months of continuous utilization of the solid injector–capillary column–macro-ECD system with derivatized extracts, the stability of the ECD background signal was excellent and repetitive isothermal analysis of large batches of samples was possible whenever required. Frequent tests of the response to ionization current increments[6] and occasional inspection of the detector cell and collecting electrode revealed no surface contamination. Partial or complete dismantling of the glass injector was required from time to time in order to remove charred deposits; the re-assembled injector usually produced severe quenching of the ECD current, which recovered its stable initial value after a few hours.

DISCUSSION

As in many similar applications, diversification of derivatives improves the validity of the assay of N-OH-2-FAA: (1) the chemical course of the two-step derivatization sequence is easier to confirm when the acyl group introduced at the second step is varied; (2) a change in chromatographic retention indices can reveal certain background peaks that might overlap with the unknown or the internal standard when biological extracts are derivatized. WCOT columns are more flexible than packed columns, as their length can be varied over a wide range. As the amount of stationary phase is low in a short WCOT column, short retention times can be obtained at unusually low temperatures (Fig. 2) with chloroacyl derivatives.

A drawback of capillary column GLC was the low capacity of the column to buffer various contaminants that inevitably are present in biological extracts, and we could not predict the effects of sampling at a high repetitive rate. This study has convinced us that a solid injector–WCOT column–macro-ECD combination, once adjusted for model compounds[3], can ensure continuing performance with derivatized biological extracts run isothermally at the rate of about 10 samples per hour.

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