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PRECISION AT THE LOW PICOGRAM LEVEL IN THE ANALYSIS OF DERIVATIZED IODOTHYRONINE STANDARDS BY CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

Previously an acceptable precision (coefficient of variation less than 10%) could be obtained only part of the time for the peak areas of derivatized iodothyronine standards, even relative to that of a structurally close internal standard, 3,5-diiodo-3′,5′-dibromothyronine, when these compounds were analyzed at the low picogram level by capillary gas chromatography with electron-capture detection. This problem has now been overcome, primarily by introducing a new design, material and conditioning of a direct vapor-injector insert. An extensive analysis under practical operating conditions of derivatized 3,5-di-, 3,5,3′-tri- and 3,5,3′,5′-tetraiodothyronine at the 5–10-pg level, involving several changes in the injection equipment and several days of injections, now gives within-day coefficients of variation ranging from 2.7 to 7.7%, 1.5 to 5.3% and 1.8 to 5.2%, respectively, for the relative peak areas of these compounds.

INTRODUCTION

In earlier work, we reported that capillary gas chromatography with fused-silica columns and electron-capture detection (GC–ECD) can be used to analyze derivatized iodothyronine standards at the pg level under both isothermal and temperature-programming conditions[1,2]. It was found that this analysis was especially facilitated by the use of short columns and high column flow-rates.

In this current paper, we present further improvements of the GC–ECD system for this analysis, mainly involving the injection step. We have shortened the length of the direct vapor injector that we used previously, changed it from glass to quartz, and modified the pre-treatment and conditioning of this injector. These and certain other changes have allowed us consistently to obtain a good precision within-day for the temperature-programmed analysis of derivatized iodothyronines at the low picogram level under practical operating conditions.

The results reported here are significant not only for the potential of capillary GC–ECD to be used for trace analysis of the thyroid hormones and their metabolites

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in biological samples, but also for the analysis of similarly difficult analytes by this approach. The derivatized iodothyronines are unusual solutes for analysis by GC–ECD due to their high molecular weights (up to 1183), polar composition and content of labile iodine atoms. Thus, the ability to establish good precision consistently at the low picogram level for the analysis of these compounds by capillary GC–ECD indicates an expanding role for this method in biological analysis.

EXPERIMENTAL

Instrumentation

The instrument was a Model 3740 gas chromatograph fitted with a quartz direct vapor-injection insert (0.9 mm capillary bore, 11 cm length), which was cleaned and silanized (as indicated below) before installation; a fused-silica capillary column (10 m × 0.25 mm I.D., DB5; J & W Scientific); a constant-current, pulse-modulated, ^{63}Ni electron-capture detector, and a pressure regulator. A Varian Model 101 data system was used. The injector and detector temperatures were 300 and 320°C, respectively. The column was initially held at 200°C for 4 min, and then its temperature was raised at a rate of 30°C min^{-1} to a final temperature of 275°C, which was held for 2 min. The carrier and make-up gas was nitrogen, with flow-rates (measured at room temperature and uncorrected) of 5.0, 5.0, and 5.0 cm^3 min^{-1} in the column, at the detector insert base, and at the detector base, respectively. The septum-purge flow-rate was in the range of 2 cm^3 min^{-1} per 5 p.s.i. column-head pressure. Injections into the gas chromatograph were made with silanized 10-μl syringes, type 1701N (Hamilton Co.), fitted with a type 26S needle.

Treatment of inserts

The quartz inserts were cleaned, silanized and conditioned as follows: (1) soak in warm (50–60°C) nitric acid overnight, and rinse with water; (2) soak in warm, 6 *N* hydrochloric acid overnight, sonicate in this same solution for 10 min, and pull through 200 ml each of boiling water and methanol; (3) dry at 240°C overnight under high vacuum; (4) place on a hot-plate and solution-silanize with a warm solution of 5% each of hexamethyldisilazane (HMDS) and trimethylchlorosilane in toluene (freshly prepared), involving three changes of this solution in the interior volume of the insert over a 0.5-h period; (5) wash with warm toluene, methanol, and toluene, and dry at 240°C overnight under high vacuum; (6) vapor silanize as described previously[3]; (7) either store sealed in a desiccator until use, or install into the gas chromatograph; (8) obtain stable base frequencies in the gas chromatograph in 50°C increments up to a column temperature of 300°C, then return the column temperature to 200°C; (9) inject a mixture of the derivatized iodothyronines, approximately 250–400 pg of each, in 1 μl of toluene under the above temperature-programming conditions; (10) reduce the pressure to 5 p.s.i., and the oven temperature to 150°C, and slowly inject (over 5 sec) 2 μl of 5% HMDS solution in toluene with the needle not fully inserted (by 2 cm) into the insert, followed by temperature programming at 20°C min^{-1} up to 300°C with a 12-min hold; and (11) combine equal volumes of the solutions used in steps (9) and (10), and inject 2 μl of this mixed solution of HMDS and derivatized iodothyronines using the conditions in step (10), and then repeat step (9). The column was kept at 170°C during overnight periods between analyses, at a

flow-rate of about 2 cm^3 min^{-1}, without any changes in the injector or detector temperatures. Each morning, the GC–ECD was reconditioned according to steps 9 to 11.

The N,O-diheptafluorobutyryl methyl ester derivatives of 3,5-diiodothyronine (T_2), 3,5,3′-triiiodothyronine (T_3), 3,5-diiodo-3′,5′-dibromothyronine (Br_2T_2), and 3,5,3′,5′-tetraiodothyronine were prepared as described elsewhere[4].

The other experimental details were as defined previously[1].

Although we observed a linear range in our previous work of 0.4 to 700 pg for the analysis of derivatized iodothyronine standards by capillary GC–ECD, along with a detection limit of 30 fg[1], several parameters in the system remained to be more fully characterized and optimized, particularly in regard to the injection step.

Syringes

Two types of syringes were used for injecting the samples into the GC–ECD in our previous work, a Hamilton type 701N (fixed needle) syringe, and a type 1701RN (removable needle) syringe; only the latter has a PTFE-tipped plunger. We now prefer to use a type 1701N syringe (fixed needle, PTFE-tipped plunger). This syringe is less prone to blockage from septum particles (for undefined reasons) than the 1701RN syringe, is less subject to contamination than this latter syringe (probably because the 1701RN syringe has a more complex internal construction), and is less subject to the leakage problems of the latter RN syringe during cleaning and injection. The problem of leakage with the type 1701RN syringe increased with continued use, and involved the hub of the syringe.

Pneumatics

We have employed both a flow regulator and pressure regulator to control the carrier gas in our GC–ECD instrument, and favor the pressure regulator for the temperature-programmed analysis of our solutes. This latter regulator, as opposed to the flow regulator, gives a much shorter delay time when its setting is changed. This faster response of the pressure regulator may explain the more constant retention times that it provided for our solutes. Variation in retention times related to the pneumatics could arise from leaks at the septum during the injection step, leaks at the graphite–metal–glass connections in the system caused by repeated temperature programming, and flow-rate changes in the column accompanying this temperature programming. The pressure regulator, because of its faster response, may have given more constant retention times by responding more rapidly or consistently to these carrier-gas variations.

Injection conditions and technique

The performance of the injection step in capillary GC can be affected by several subtle parameters, such as smooth *vs.* jagged column edge[5], injection speed[6], hot *vs.* cold needle injection[7], and needle-residence time[8]. We examined all of these factors in our temperature-programmed analysis of derivatized iodothyronines, and did not observe any major changes in the resultant chromatograms. This included the solute-peak shapes, chromatographic response and short-term (five injections) precision in these chromatograms, which never changed by more than 25% (although the solvent peak is considerably broader when a slow injection is carried out). When lindane and

aldrin were injected under isothermal conditions, hot-needle injections gave peak heights as much as 1.7 times higher than those obtained by injection with the cold-needle technique.

The use of a longer needle (3 in. as opposed to the customary 2 in.) was also examined, potentially to minimize any back-flashing, and reduce the exposure of the sample vapor to the walls of the injector insert. However, the result was a reduced response (about 2-fold) for our solutes, with no improvement in short-term precision, perhaps due to increased exposure of the sample components to the outer syringe-needle surface during the injection step.

Injection inserts

Our standard practice previously was to replace the glass insert in our injector whenever a change in peak shape was observed for our solutes that could not be traced to other causes. The average lifetime of the inserts was 2 to 4 weeks when derivatized iodothyronines were analyzed above the 100-pg level. However, when we began repeated injections of these solutes at the 5–10-pg level, we observed inconsistent performance from one insert to another, even when apparently equivalent inserts were cleaned and silanized together before installation. Some inserts performed equivalently (in terms of chromatographic response, peak shapes, and insert lifetime) whether 5–10-pg, or 100-pg or larger amounts of the derivatized iodothyronines were injected repeatedly. Other inserts gave a good performance only for injections of these solutes in amounts above 100 pg. For these latter inserts, significantly decreased responses, or even distorted chromatograms, were observed for the injection of low picogram amounts of these solutes. In all instances, satisfactory peak shapes and responses were observed for the injection of low picogram amounts of lindane and aldrin. These latter substances are relatively inert as solutes when analyzed by GC–ECD.

In some of the distorted chromatograms, we observed a mixture of sharp and broad solute peaks, suggesting that the cold trapping of the solutes during the injection step might be occurring on two types of adsorption sites. Since the injector in our instrument is partly exposed to the column oven, it was likely that cold trapping of the solutes was occurring in both the lower part of the injector insert, and at the top of the column, during the injection step. Glass-lined metal inserts are available from the manufacturer for the injector on this instrument, and these types of inserts were tried in an effort to maintain the overall injection insert at a high temperature, including the part that is exposed to the lower temperature of the column oven during the injection step. Although the initial performance of these inserts was satisfactory, the structure and presence of the metal sleeve limited the strength of the nitric acid cleaning conditions that could be employed. The final result was that we were unable to re-use these inserts once they had been contaminated with 3 to 4 weeks of injection residues in the instrument.

Based on this experience, we shortened the injector insert, changed it from glass to quartz, and subjected it to more extensive silanizing and conditioning steps, as described under Experimental. We then proceeded to analyze the derivatized iodothyronines repeatedly with temperature programming by GC–ECD. Overall, we made 217 analytical injections of these solutes at the 5–10-pg level, and 10 such injections at the 0.5–1.0-pg level, averaging 15 injections per day, and comprising 4

successive inserts and 15 days of injections. The resultant data were calculated as follows for the 5–10-pg level. First, we determined the ratios of the peak areas and heights for derivatized T_2, T_3 and T_4 relative to the corresponding areas and heights for the internal standard, derivatized Br_2T_2. Then we averaged these values on each day. The resultant within-day means and coefficients of variation (C.V.) were grouped in terms of the 4 inserts employed, and the lowest and highest mean and C.V. values in each group were selected for presentation here, as seen in Table I.

TABLE I

PRECISION OF PEAK RATIOS FOR ANALYSIS OF DERIVATIZED IODOTHYRONINES AT THE 5–10-pg LEVEL

Insert/days in use/total No. of injections per insert	*Within-day mean values and C.V. values for peak ratios**					
	Range of area values (and C.V., %) for each insert			*Range of height values (and C.V., %) for each insert*		
	T_2/Br_2T_2	T_3/Br_2T_2	T_4/Br_2T_2	T_2/Br_2T_2	T_3/Br_2T_2	T_4/Br_2T_2
A/2/38	0.80–0.81 (3.8 –7.7)	0.72–0.73 (4.0 –5.3)	1.24** (2.2)	0.83–0.84 (7.7 –9.3)	0.86–0.87 (6.6 –8.8)	(0.80)** (3.9)
B/6/98	0.88–0.98 (2.6 –6.9)	0.73–0.83 (1.9 –4.8)	1.11–1.22 (1.8 –5.2)	0.86–1.04 (4.7 –7.6)	0.84–0.95 (3.6 –7.0)	0.70–0.81 (4.2 –6.4)
C/5/72	0.84–0.86 (2.7 –6.3)	0.79–0.81 (1.5 –2.8)	1.20–1.25 (2.1 –3.7)	0.75–0.83 (3.4 –6.6)	0.88–0.91 (2.9 –5.8)	0.72–0.91 (2.5 –4.5)
D/2/9	0.73 (4.3)	0.70 (3.7)	1.21 (2.0)	0.69 (5.3)	0.75 (7.1)	0.80 (4.0)

* On each day, an aliquot of the same stock solution (kept at 4°C) of derivatized iodothyronine standards in toluene (228, 204, 253 and 437 ng/ml of derivatized T_2, T_3, Br_2T_2 and T_4, respectively), was diluted with toluene to the 5 to 10 pg/μl level. This working solution was kept at room temperature and injected throughout the day.

** Outlying mean peak-area and peak-height values of 1.60 and 1.05 were observed for T_4 on the first day of the experiment, apparently due to adventitious contamination of this solution with derivatized T_4, since this ratio persisted for this working solution on the second day, whereas a fresh working solution gave an acceptable ratio for T_4/Br_2T_2.

Precision at the 5–10-pg level

The main point to be drawn from the data in Table I is that an acceptable level of precision (C.V. values ranging from 1.5 to 7.7% for peak-area ratios, and from 2.5 to 9.3% for peak-height ratios) is established for this analysis on any given day, irrespective of the degree of variation that develops in the mean peak ratios when an insert is changed, or between days for a given insert. Changes in the mean values for the peak areas and heights arising between analyses conducted with different inserts, and determined for each derivatized iodothyronine, range from 13 to 34%, and from 27 to 51%, respectively. (For example, 0.98 − 0.73/0.73 = 34% as the change in the mean peak-area ratios for derivatized T_2). Between-day variations for a given insert were smaller, ranging from 9 to 14% for the mean peak-area ratios, and 13 to 26%

for the mean peak-height ratios. Closer analysis of the original data shows random variation to be present as opposed to any drift in the values on a given day. Assuming that these variations arise from active sites in the GC–ECD system, we see progressive differences in these sites when the results are compared day-to-day, and then insert-to-insert.

The precision for the relative peak areas is better than the precision for the relative peak heights by an absolute difference of about 1–2% overall. Considering that the C.V. values for the peak heights occasionally are actually lower than the corresponding C.V. values for the areas, the main conclusion on this aspect is that only slightly better precision is obtained by measuring peak areas (with the data system employed) as opposed to peak heights (manually) in this analysis.

Performance of the internal standard

It is next interesting to compare the relative monitoring of the three solutes, T_2, T_3 and T_4, by Br_2T_2, the internal standard (all derivatized). From a structural standpoint, Br_2T_2 would seem to be most similar to T_4, but the retention of Br_2T_2 is closest to that of T_3. Consistent with this, the overall variations in peak ratios and in C.V. values, either for areas or heights, are comparable for T_3 and T_4, but somewhat higher for T_2. Thus, a more appropriate internal standard for T_2 may allow closer monitoring of this solute.

Precision at the sub-pg level

For the ten analyses conducted at the 0.5–1.0-pg level, which involved five injections each on separate days (one day with insert C, and one with D), the C.V. values for the mean peak-area ratios ranged from 8.0 to 9.6% with insert C, and from 5.1 to 5.5% with insert D (data not shown in Table I). Thus, an acceptable level of precision is obtained, at least initially, at this lower solute level as well. A representative chromatogram from one of these analyses is shown in Fig. 1.

The corresponding values for the mean peak-area ratios of these solutes at the 0.5–1.0-pg level were 0.78, 0.88 and 1.13 for T_2, T_3 and T_4, respectively, with insert C, and, correspondingly, 0.62, 0.62 and 1.30 with insert D. These mean values differ from the mean values for the peak-area ratios established on the same day at the 5–10-pg level for these compounds by −7, +11 and −6% for T_2, T_3 and T_4, respectively, with insert C, and by −15, −10 and +8% in a corresponding manner with insert D on the other day involved. This is consistent with our prior conclusion that the linear range for the derivatized iodothyronines with the current equipment and techniques is 0.4 to 700 pg, with some indications of reproducible non-linearity (agreement among triplicate injections) near the level of 0.4 pg (see ref. 1). It further now appears that this non-linearity, although consistent within-day, tends to vary, at least for analyses conducted with different inserts prepared according to the current method.

Detector make-up flow-rates

The manufacturer recommends a total flow-rate of 30 cm^3 min^{-1} in the GC–ECD that we use. Consistent with this recommendation, we observed the lowest baseline noise level under this condition, as obtained for example, by column, column make-up, and detector make-up flow-rates of 5.0, 20.0,, and 5.0 cm^3 min^{-1}, respec-

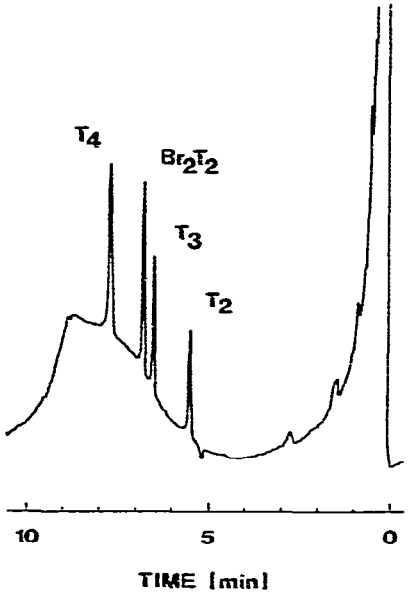

Fig. 1. Analysis of derivatized T_2, T_3, Br_2T_2 and T_4 at the 0.5 to 1.0-pg level by capillary GC–ECD with temperature programming; attenuation 80.

tively. However, similar settings of 5.0, 10.0, and 5.0 cm^3 min^{-1} gave a 2-fold higher response with only a 25% increase in noise, and settings of 5.0, 5.0 and 5.0 cm^3 min^{-1} provided an additional 20% higher response with only a 30% further increase in noise (measured at attenuation 2). The latter as opposed to the initial conditions were selected for the experimental work reported in this paper, since baseline noise was not evident at the attenuation value of 128 generally employed here, while a 50% reduction in carrier-gas consumption was realized.

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