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LARGE INJECTION VOLUMES OF DERIVATIZED IODOTHYRONINES IN CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

The response of some derivatized iodothyronines by capillary gas chromatography with electron-capture detection decreases with larger injection volumes under isothermal conditions (column at 265°C). However, this response is constant under temperature-programmed conditions (column initially at 200°C). These results are not influenced by the different retention times of these solutes, nor by the length or composition of the injector (glass *vs.* quartz, each silanized), but are influenced significantly by the choice of injection solvent. Collectively, these data indicate a solvent-induced loss of the derivatized iodothyronines in the fused silica column, as opposed to a loss in the injector, or a loss (or decreased sensitivity) in the electron-capture detector, with larger injection volumes. Nevertheless, the chromatographic performance, aside from the lower recovery, is acceptable even with injection volumes as large as 6 μl under isothermal conditions onto a narrow-bore capillary column, due primarily to the high flow-rate of carrier gas in the injector and column (11.5 cm^3 min^{-1}), and the characteristics of the derivatized iodothyronines in this type of analysis.

INTRODUCTION

The volume of sample injected into a gas chromatograph fitted with a capillary column is usually kept small in order to minimize the initial width of the sample on the column, thereby optimizing the efficiency of the solute peaks and minimizing the overlap of these peaks with the solvent tail[1]. However, it can be advantageous, particularly in trace analysis, to inject larger volumes. This is because the use of such volumes may enhance the recovery and convenience of the final steps in sample handling, such as redissolving or extraction/washing steps, prior to injection of the sample into the gas chromatograph.

In our work on the trace analysis of derivatized iodothyronines by capillary gas chromatography (GC), we have been evaluating the injection of large sample volumes into the instrument. For example, previously we reported that a direct sampling injection technique (which places all of the injected sample onto the column), along with temperature programming, allows a constant response to be obtained for

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derivatized iodothyronines even when increasing volumes up to 8 μl are injected onto a narrow-bore capillary column[2]. Nevertheless, we noted that a decreased response is obtained under isothermal conditions (involving a higher column temperature) with such larger volumes.

In this paper we more thoroughly investigate this decreased response with larger injection volumes under isothermal conditions for derivatized iodothyronines. Based on the results from several experiments, we conclude that the lower response is due to a loss of these solutes in the fused-silica column, rather than in the injector or detector of the instrument. Excluding this loss in response, we observe that this system is quite tolerant of larger injection volumes.

EXPERIMENTAL

The derivatized iodothyronines were synthesized as described previously[3]. The instrument was a Model 3740 gas chromatograph fitted with a glass direct injection insert (Varian, 15.5 cm × 0.9 mm I.D.), or a quartz insert (11 cm × 0.9 mm I.D.) each of which was silanized before installation[2]; a 15-m fused-silica capillary column (0.25 mm I.D.), either as a DB5 column (a bonded version of SE-52) or coated with a 0.25-μm film thickness of SE-52 (J and W Scientific); and a constant-current, pulse-modulated, ^{63}Ni electron-capture detector (ECD). The injector, column, and detector temperatures were 270, 265, and 320°C, respectively, for the isothermal analyses. For the analyses under temperature-programmed conditions, the same temperatures were used for the injector and detector, but the column was initially held at 200°C for 2 min, and then its temperature was raised at an actual rate of 43°C min^{-1} (from a setting of 60°C min^{-1}) to a final temperature of 275°C. The carrier and makeup gas was nitrogen with an actual flow-rate in the column of 11.5 cm^3 min^{-1} at 265°C, and flow-rates of 10 and 8.5 cm^3 min^{-1} (measured at room temperature and uncorrected) at the detector base and detector insert base, respectively. The septum purge flow-rate was 2 cm^3 min^{-1} per 5 p.s.i. column head pressure, and the head pressure was 30 p.s.i. Injections into the gas chromatograph were made with silanized 10-μl syringes, either type 1701N or 1701RN (Hamilton), fitted with a type 26S needle.

RESULTS AND DISCUSSION

Characteristics of derivatized iodothyronines

The compounds analyzed in this work are derivatized iodothyronines, and their structures are shown in Fig. 1. In the underivatized form, T_3 and T_4 are the two naturally occurring thyroid hormones, T_2 is a deiodination metabolite of these hormones, and Br_2T_2 is a synthetic analogue which has been used as an internal standard in the analysis of these substances by GC–ECD[4].

The iodothyronines are present in trace amounts in physiological samples, and they require derivatization prior to analysis by GC. Large injection volumes, therefore, can facilitate the analysis of these substances by allowing larger volumes during the steps immediately preceding injection of the sample into the gas chromatograph, for higher recoveries and more convenience.

The derivatized iodothyronines are somewhat unusual as solutes for analysis

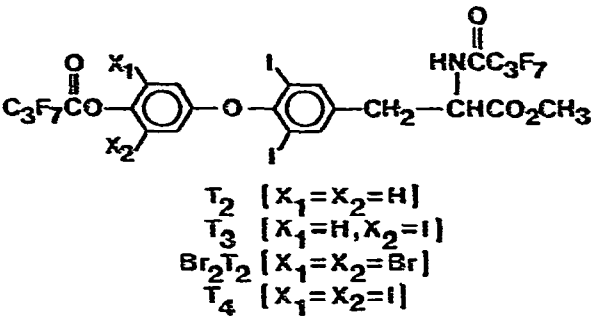

Fig. 1. Structures of the derivatized thyroid hormones.

by GC, primarily because of their high molecular weights (range 600–1200) and polar composition. Consequently, they possess limited thermal stability, and their recovery is optimized by increasing the flow-rate in the column[2]. Further, the plate numbers observed for their peaks are significantly lower, *e.g.* by three- or four-fold, than the corresponding plate numbers for the peaks of simple solutes such as lindane, under the same flow-rate conditions.

Use of large injection volumes

The conditions of broad peaks, solutes with low volatility, and a high flow-rate of carrier gas potentially create a special opportunity to inject larger sample volumes even with a direct sampling technique and a narrow-bore column under isothermal conditions without a significant change in chromatographic performance. There are several reasons for this: (1) broad peaks are less susceptible on a relative basis to further broadening from injection effects; (2) the high flow-rate and accompanying high inlet pressure will act to minimize the back diffusion as well as backflashing during the injection step; and (3) the larger solvent volumes will tend to focus the solutes at the beginning of the column[5]. In regard to the first reason, we observe a peak width for T_4 of 53 sec with a sample injection volume of 0.5 μl at a flow-rate of carrier gas in the column of 11.5 cm^3 min^{-1}. In the ideal case, where the effective sample volume upon injection is the vapor volume of the sample at the injector temperature and pressure, we calculate that even a much larger injection volume (*e.g.* 4.0 μl) would require only 4.3 sec to be swept into the column. This particular circumstance, aside from the other effects noted, would add very little to the initial peak width obtained with a 0.5-μl injection volume. Also, the high retention of these compounds, relative to the elution time of the solvent peak, and the selectivity of the electron capture detector, tend to significantly displace the peaks for these compounds from the solvent tail, further minimizing the potential influence of a larger injection volume on the chromatogram.

To pursue this experimentally, we injected larger sample volumes by diluting a given amount of solute with more solvent prior to injection (constant solute mass for each of a series of injections), and we also injected increasing volumes of a given solution (increasing solute mass with each injection). In both cases, consistent with the above discussion, the band width at half height, *e.g.*, for derivatized T_4, only increased by 18% even up to an injection volume of 7.0 μl. The peak asymmetry was

constant (at a value of 2.0 for derivatized T_4) up to an injection volume of 4.0 μl, but increased two-fold for an injection volume of 6.0 μl and four-fold for 7 μl.

Unexpectedly, however, the response for these solutes decreased in each of these experiments with larger injection volumes. This is shown in terms of peak height for constant solute mass with larger injection volumes in Fig. 2, and for increasing solute mass with larger volumes in Fig. 3. For example, as seen in Fig. 2, the response falls off *ca.* two-fold when the original sample volume of 0.5 μl is diluted to 4.0 μl prior to injection.

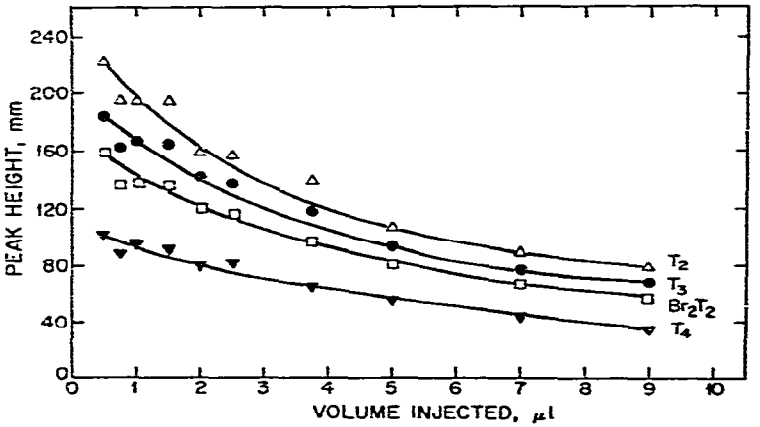

Fig. 2. Peak height for derivatized T_2, T_3, T_4 and Br_2T_2 with constant solute masses (195, 326, 310 and 301 pg, respectively) as a function of injection volume at a flow-rate of 11.5 cm^3 min^{-1}.

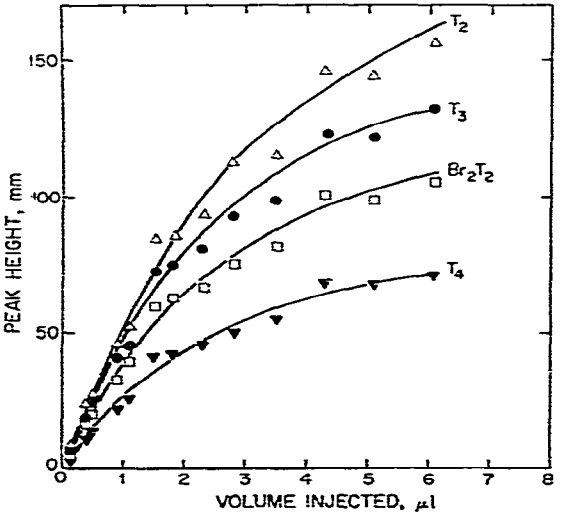

Fig. 3. Peak height for derivatized T_2, T_3, T_4 and Br_2T_2 as a function of increasing solute masses from larger injection volumes of a solution containing 78, 130, 124 and 120 pg μl^{-1}, respectively, at a flow-rate of 11.5 cm^3 min^{-1}.

Source of decreased response

The potential sources of the lower response with larger injection volumes are either a decreased recovery in the injector, column or detector; or a decreased sensitivity in the detector due to the presence of pre-eluting or co-eluting injection solvent.

The decrease in response with larger injection volumes is quite unlikely to arise in the detector from a decreased sensitivity due to the injection solvent, since a parallel decrease in the response for all of the derivatized iodothyronines is observed, in spite of their markedly different retention times, as the volume of injection solvent is increased from 1 to 9 μl. (This progressively subjects these solutes differentially to the presence of injection solvent in the detector.) For example, the coefficients of variation for the peak height ratios of derivatized T_2, T_3 and T_4 relative to Br_2T_2 as a function of injection volume, are 4.4, 2.3 and 4.4%, respectively, for the data in Fig. 2, and range from 2.0 to 4.5% for the data in Fig. 3. This also makes it extremely unlikely that a solvent-induced loss of these compounds is occurring in the detector.

The injector temperature was 270°C in this work, the same as in our previous experiments with temperature programming (2–8 min hold at 200°C, program up to 275°C) in which a constant response was obtained with increasing injection volumes[2]. Ordinarily this would rule out the injector as the source of the decreased response. However, in this gas chromatograph, the lower end of the injector is located in the column oven, so that the temperature of the injector is not uniform throughout its entire length when the temperatures of the injector compartment and column oven are different.

To fully exclude the injector as the source of decreased response with larger

TABLE I

RESPONSE OF THE DERIVATIZED IODOTHYRONINES, AS A FUNCTION OF THE INJECTION VOLUME, INJECTION SOLVENT, AND THE TEMPERATURE CONDITIONS APPLIED TO THE COLUMN

Column and injection conditions		*Derivatized iodothyronines: peak area per microliter of injection volume**			
		T_2	T_3	Br_2T_2	T_4
Temperature programmed					
Toluene	1 μl	1.00	1.00	1.00	1.00
	6 μl	0.92	1.05	1.08	0.88
Acetonitrile	1 μl	0.52	0.58	0.60	0.55
	6 μl	0.60	0.69	0.56	0.60
Isothermal					
Toluene	1 μl	0.93	1.06	0.98	0.92
	6 μl	0.38	0.54	0.52	0.54
Acetonitrile	1 μl	0.16	0.29	0.22	0.29
	6 μl	—**	0.35	0.26	0.30

* In each case, a mixture of 72, 70, 107 and 76 pg, respectively, of derivatized T_2, T_3, T_4 and Br_2T_2 was injected onto a DB5 column, in an injection volume of either 1 or 6 μl. The resulting peak areas are normalized relative to the areas obtained by injecting 1 μl of a solution of the derivatized iodothyronines under temperature-programmed conditions, as shown.

** The peak for derivatized T_2 was obscured by the solvent peak under these conditions.

injection volumes, we not only insulated and shortened it (from 15.5 to 11 cm), so that it was no longer partly exposed to the column oven, but we also changed its composition from glass to quartz (each of which was silanized). Then we repeated the injection volumes of 1 μl and 6 μl of the derivatized iodothyronines in toluene as before, using both isothermal and temperature-programmed conditions. As seen in Table I, the response is essentially constant for these compounds only with temperature programming, and essentially the same decrease in response is obtained as observed in Fig. 2 and 3 under isothermal conditions with the longer, non-insulated, silanized glass injector. Thus, the injector clearly is not the source of the decreased response under isothermal conditions.

By a process of elimination, this implicates the column as the site of the decreased response with larger injection volumes when its temperature during the injection step is 265°C. If this, indeed, is the case, then one might expect that the recovery of the derivatized iodothyronines would be influenced by the nature of the injection solvent. Thus, we compared the response with acetonitrile rather than toluene as an injection solvent, both for large and small injection volumes and under both isothermal and temperature-programmed conditions.

These results are also summarized in Table I, and were obtained with the modified injector (shortened, insulated, and composed of silanized quartz). For all injection volumes under both isothermal and temperature-programmed conditions, we see that the response is significantly lower with acetonitrile than with corresponding volumes of toluene. Other comparisons can be made as well between the results from these two solvents as a function of the temperature conditions and injection volume, but the main point for our purposes is simply that the response is markedly affected by the nature of the injection solvent.

Thus, the decreased response is related not only to the column temperature but also to the volume and type of injection solvent. These results collectively indicate that the decreased response with larger injection volumes arises from a loss of the derivatized iodothyronines in the column as opposed to losses or decreased sensitivity elsewhere in the GC–ECD. The practical significance of these results is to indicate the potential for temperature or solvent related effects on the response to complex solutes even in a deactivated, fused-silica column. Consistent with this, previously we observed that column temperatures in excess of 265°C during the injection step lead to losses of these compounds on the column[2]. We suggested that these losses were thermal rather than adsorptive in nature, and the current data now allow the elaboration that the injection solvent may be contributing to these former losses with higher temperature as well. Further, it is conceivable that the changes in response with higher flow-rates under isothermal conditions were similarly related, in part or in total, to the presence of the injection solvent in the injector or the column.

Performance with large injection volumes

The chromatogram resulting from the injection volume of 6 μl in the previous experiment involving increasing solute mass (Fig. 3) is shown in Fig. 4. Obviously, the chromatographic performance is acceptable in spite of the total injection of an unusually large volume of sample under isothermal conditions onto a narrow-bore column. Thus, this approach potentially can be utilized to facilitate the trace analysis of these compounds as discussed above. Although column lifetime potentially may be short-

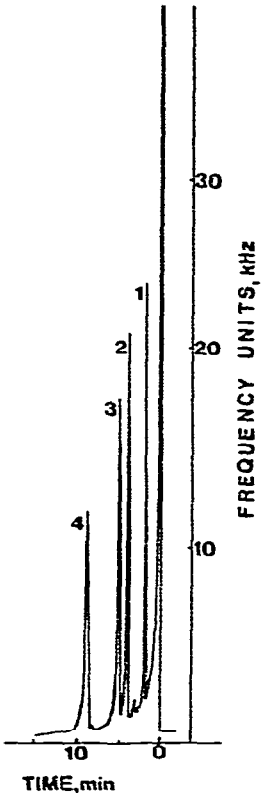

Fig. 4. Chromatogram corresponding to an injection volume of 6 μl in Fig. 3. Peaks: 1 = T_2; 2 = T_3; 3 = Br_2T_2; 4 = T_4.

ened by leaching of the liquid-stationary phase by large volumes of injection solvent, bonded phases should tend to resist this problem.

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