GLC Assay of Sodium Thyroxine

Keyphrases ☐ Thyroxine, sodium—analysis ☐ Diazomethane–thyroxine reaction—thyroxine analysis ☐ Estradiol benzoate—GLC internal standard ☐ GLC—analysis

Sir:

In the course of evaluating chemical assays of thyroid principles, it became clear that GLC held promise of reliable assays which would be simple, rapid, and highly specific. This communication reports our initial experience with a useful derivative of thyroxine prepared by reaction with diazomethane in a mixed solvent of methylene chloride and dimethyl sulfoxide under ambient conditions of temperature, pressure, and moisture.

Several groups reported other derivatives of thyroxine which allowed successful chromatography, and these are outlined here. Based partly on experience with several of those derivative sequences, we concluded that an alternative sequence was necessary. We wanted a one-step reaction in a simple vessel that would be relatively insensitive to other materials present, particularly moisture and salts. Because the USP and the NF list the sodium salts[1] of the optical isomers of thyroxine, the sequence had to be directly applicable to the sodium salt so that monograph purity and identity tests and assays could be developed. Methylation with diazomethane is commonplace for acids and has been used with phenols to a more limited extent.

The following sequence illustrates the methodology. Weigh 8 mg. of undried sodium *d*- or *l*-thyroxine into a narrow tube[2] and add 0.1 ml. dimethyl sulfoxide[3] containing aqueous HCl, 5% by volume. Solution is rapid. Add 1.0 ml. chromatographic grade methylene chloride containing 1.00 mg. estradiol benzoate NF reference standard as the assay internal standard. At this point, fine droplets may appear which consist of sodium chloride and any water present. In a hood, add 1.0 ml. diazomethane reagent[4], about 0.6 *M* in 2:1 ether–methylene chloride prepared by distillation of ethereal diazomethane into methylene chloride. Mix and allow to stand 30 min. using a polyethylene stopper. Uncover and evaporate the solution to a volume of about 0.2 ml. in a stream of N_2. The tube may be placed in warm water to speed the evaporation. Add 1 ml. methylene chloride and mix. A clear, colorless solution is obtained, containing a fine precipitate of salt. Inject 1.5 μl. or about 12 mcg. of derivative, on-column, in the following system: 0.6 m. × 4 mm. i.d. glass column, packed with 3% OV-1 on 80/100 silanized, acid-washed, flux-calcined diatomite[5], no-flow cured at 340° for 4 hr., and conditioned at 280°; helium carrier gas, 80 ml./min.; column temperature, 250°; and detector and injection port, 260°. The methylated T_4 derivatives elute in about 10 min., with a relative retention of 1.82 and 2.07, based on estradiol benzoate. The peak area of the methylated T_4 pair, the internal standard, and any other peaks are measured, and the ratio of areas of methylated T_4/estradiol benzoate is calculated. Peak height data are not acceptable.

Samples of sodium thyroxine, corrected for loss on drying, are compared quantitatively by this procedure, using a three-point calibration curve based on duplicate 6-, 8-, and 10-mg. samples of an arbitrary standard batch of either the salt or free acid. Moderate precision, 1.08%, was obtained without planimeter on multiple injection of a single 8-mg. sample. The column used to obtain the precision data gave a rectilinear calibration curve for the sample concentrations of 4, 6, 8, and 10 mg. Duplicates were within 1%.

For chromatographic purity, an internal standard is not added and suitable changes in electrometer attenuation are made to allow calculation of area percent of all peaks. Liothyronine (T_3) elutes as a pair of peaks at relative retention of 0.82 and 0.93; diiodothyronine occurs at 0.48; diiodotyrosine elutes too rapidly, 0.07, for accurate quantitation in this isothermal system, so that where this substance is important, temperature programming is necessary. The latter peak of T_3 predominates with longer reaction times and is the only peak after 5 hr. Slower methylation of the T_3 phenol compared to T_4 is consistent with the lesser acidity, *i.e.*, pKa 8.4 compared to pKa 6.6 (1). Tetraiodothyroacetic acid gives a peak at relative retention 1.02, but this offers no advantage as an internal standard, other than reaction control, over the steroid ester which is available in a controlled form.

Methylation of T_4 leads to a major peak, which is 93% of the total area, and one less mobile peak. The major peak exhibits no tailing and a symmetry of 0.93 due to the presence of another minor, but more mobile, methylated T_4 species. Initial NMR observations on the analogous diiodotyrosine suggest complete methylation of both phenolic and acid hydroxyls with possible methylation of the amine. Removal of excess diazomethane prior to injection is necessary to prevent side-product formation.

The column reported here, about 0.75 m., gave 1100 apparent plates for methylated T_4 and 1300 plates for estradiol benzoate with a 1.2 tailing factor. A column yielding 1000–1200 plates for estradiol benzoate, with a tailing factor less than 1.3, would be presumed adequate for this assay because the steroid is more chromatographically demanding than any of

[1] *Levo*-isomer as a thyroid hormone and *dextro* as an antihypercholesterolemic.

[2] Small centrifuge tubes or screw-cap vials. Silane treatment is not essential.

[3] Spectroquality, N_2 sparged.

[4] Diazald, Aldrich Chemical Co., prepared as directed except as noted.

[5] Gas-chrom Q and Supelcoport were used, both self-coated and supplier coated.

the iodoaminoacids. Samples as small as 1 mcg. of the methylated T_4 are symmetric, but the secondary peak is relatively larger. At higher sample sizes, greater than 20 mcg., severe front-edge leading develops, and this reflects the unusual solubility relationships of thyroxine. Linear calibration lines passing nearly through zero can be prepared by injection either of varying amounts of a single sample (1–30 mcg.) showing negligible, nonlinear adsorptive losses or by varying sample sizes of sodium thyroxine over a final sample concentration equivalent to 1–18 mg./ml. thyroxine. Using a 3% liquid coating, optimum chromatography requires injection of 5–20 mcg. of thyroxine. Smaller samples are best analyzed on 1% coatings, but caution is necessary. In our hands, a 1% OV-1 column showed distinct overload effects with samples in excess of 5 mcg. of thyroxine. This 1% loading allowed the detection of less than 50-ng. samples using flame ionization. Thermal instability causes significant losses, 24% at 340°, when very high injection port temperatures are used.

Flame-ionization detector (FID) response is notable. On a mass basis alone, the relative area response of methylated T_4 is only 15.7% that of estradiol benzoate. The response of the FID is commonly related (2) to the carbon content of the species, specifically those carbons not bonded to heteroatoms. The methylated T_4 response relative to the steroid when corrected for carbon content of the molecules is 47%. The interference of the heteroatoms, particularly the iodo groups, with the usual FID response is obvious.

Other reaction systems were investigated. Derivatization in plain methylene chloride led to a different peak pattern, with the minor, more mobile, methylated species becoming more abundant. Reaction of the phenol was slower, and initial solution of samples was less reliable. Although reaction in 40% dimethyl sulfoxide in methylene chloride led to the usual peak pattern, methylated T_4 is less soluble in dimethyl sulfoxide than in methylene chloride, so that calibration curves were not possible using concentrations above 4–5 mg./ml. in this solvent. The reverse holds for sodium thyroxine, which is directly soluble in dimethyl sulfoxide to at least 80 mg./ml. but which is only slowly soluble in methylene chloride. Solutions of the salt are yellow-brown. The methylated derivative, however, is soluble in methylene chloride and gives linear calibration curves up to at least 18 mg./ml. These solubility relations are the key to reliable quantitation of sodium thyroxine by GLC. A polar solvent, dimethyl sulfoxide, is necessary to dissolve the sample crystals; acid is necessary to convert the salt to the free acid which is soluble both in dimethyl sulfoxide and the much less polar 10% dimethyl sulfoxide–methylene chloride. Methylation leads to a derivative that is soluble in methylene chloride but which is much less soluble in polar solvents such as dimethyl sulfoxide and also in ether. Methylene chloride appears to be the solvent of choice for methylated thyroxine.

Dimethyl sulfoxide is desirable to dissolve the sodium salt, but the final concentration in the sample should be kept low for several reasons: diminished solubility of the derivative and the adverse effects of high-boiling solvents on reproducibility of elution data and analysis of rapidly eluted solutes. The effect of 40% dimethyl sulfoxide solvent on methylated T_4 was to make peak heights and retention times highly variable, so that the standard procedure, even with the more reproducible 10% dimethyl sulfoxide solvent, is to measure only peak areas. Formic or acetic acids in place of hydrochloric acid gave incomplete reaction. Direct reaction of the sodium salt was not rapid or quantitative, and a different peak pattern developed.

Alternative liquid phases were studied. OV-61 and OV-17 consist of 2:1 and 1:1, respectively, methyl–phenyl radicals in the polysiloxane. These gave much greater resolution but required much greater temperatures and carrier flow. Solvent elution was prolonged. Both were usable as 1.5% loadings, but the OV-61 was clearly less stable over long periods of use. All columns required occasional replacement of the first 10 cm. of packing, preferably after 30–50 injections. The OV-17 system may find application in biological studies or in a study of the reaction of diazomethane with iodoaminoacids. Detection by a hot-wire katharometer failed as sensitivity decreased with each injection, presumably due to chemical reaction with the halogenated species.

Two other approaches have led to successful GLC of thyroxine. Multiple-step syntheses of *N,O*-diacyl alkyl esters were reported by Stouffer *et al.* (3), Jaakonmaki and Stouffer (4), Hagen *et al.* (5), and Richards and Mason (6). None of these workers reported quantitative aspects. Variable solubility relations in the preparation of *N,O*-bisacetyl methyl esters and the need to control several steps diminished our interest in this approach.

Silylation methods have been applied, with the apparent advantage of one-step derivatization. GLC of iodoaminoacid trimethylsilyl derivatives was reported by several groups (7–9) using several silylation reagents. None of these workers considered solubility effects or the presence of polar materials or moisture on these reactions. We were unable to obtain reproducible silylation from concentrates by evaporation from solutions containing moisture and buffer salts. Also, the limited solubility of T_3 and T_4 made the direct use of silylating reagents uncertain. Sodium T_4 did not react readily due to failure to dissolve. Backer and Pileggi (10) made the very important observation that serum extracts using either cation- or anion-exchange resin workups did not yield samples suitable for silylation. Funakoshi and Cahnmann (11) made the additional observation that the sodium salts reacted only sluggishly. The structure of the trimethylsilane derivatives of iodoaminoacids is unresolved (8, 11). Observations made by the various workers on the problems of reaction of sodium salts and the presence of polar materials were confirmed in our hands and led us to search for an alternative to silylation. Work is planned to determine the applicability of the methylated derivative to biological extracts.

Osborn and Simpson (12) recently reported a sequential derivatization scheme for the identification of ^{125}I metabolites by TLC. They combined six solvents with four reactions: acetylation, hydrolysis, methyl

esterification, and methyl etherification. Methanolic diazomethane was used to prepare methyl ethers. The scheme allowed identification of a T_3 isomer in plaice, a flatfish (*Pleuronectes platessa*).

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N^{α},N^{α}-Dimethylhistamine, the Hypotensive Principle of the Sponge *Ianthella* sp.

Keyphrases ☐ *Ianthella* sp.—hypotensive constituent ☐ N^{α},N^{α}-Dimethylhistamine—isolation, identification ☐ Hypotensive activity—N^{α},N^{α}-dimethylhistamine

Sir:

The ubiquity of histamine in nature is well known; however, the occurrence of histamine derivatives in biological systems is less well known (1–8). We wish to report the isolation and identification of the potent hypotensive agent 4(or 5)-[2-(dimethylamino)ethyl]-imidazole from the sponge *Ianthella* sp.[1]. A previous report suggested the presence of this compound in the sponge *Geodia gigas*, but insufficient data were presented to afford unambiguous structure assignment (7, 8). Isolation of N^{α},N^{α}-dimethylhistamine from aqueous extracts of the sponge *Ianthella* sp. was effected by a combination of absorption and partition chromatography. Purification was followed by hypotensive activity in the anesthetized dog preparation.

Experimental[2]—Melting points were determined with a Thomas-Hoover capillary melting-point apparatus and are uncorrected. IR spectra were determined on a Beckman model IR-8 spectrometer. NMR spectra were determined on a Varian A-60 spectrometer, using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard. Mass spectra were determined on a Hitachi-RMU-6H mass spectrometer.

Isolation of 4(or 5)-[2-(Dimethylamino)ethyl]imidazole—A 100-g. sample of dried pulverized sponge *Ianthella* sp. was shaken with two 600-ml. portions of water for 2 hr. The two fractions were combined, and water was removed under reduced pressure at a temperature less than 50°. The residue was triturated with two 600-ml. portions of absolute ethanol. Combination of the fractions and removal of solvent gave 9 g. of residue, which had appreciable activity as a hypotensive agent.

Chromatography—The residue from initial extractions (9 g.) was dissolved in a minimum of absolute ethanol and placed on a 550-g. Florisil[3] column. The column was developed with increasing concentrations of ammonium hydroxide in absolute ethanol. Column effluence was monitored both by residue weight and hypotensive activity as determined in the anesthetized dog. Material eluted with 1 and 2% ammonium hydroxide in ethanol contained the desired activity. These fractions were combined, and the solvent was removed under reduced pressure to give 1.7 g. of material. TLC (acetonitrile–ammonium hydroxide) analysis of the residue indicated one principal component.

Further purification was effected by partition chromatography in the solvent system 2-butanol–ethyl acetate–triethylamine–water (600:400:10:990) using Celite 545[4] as the column support. Fractions (10 ml.) were collected automatically. Column fractions were monitored by weight and hypotensive activity. The fractions (homogeneous by TLC) were combined, and the solvent was removed under reduced pressure to give 685 mg. of desired material.

Characterization of 4(or 5)-[2-(Dimethylamino)ethyl]-imidazole—An analytical sample prepared by molecular distillation of the active component from column chromatography had an elemental analysis consistent with the formula $C_7H_{13}N_3$. The mass spectrum was characterized by a parent ion peak at *m/e* 139 with additional principal peaks at 124, 95, and 81 mass units. The NMR spectrum (D_2O) exhibited a sharp 6-proton absorption at δ2.19 (CH_3), a 4-proton absorption at δ2.65 (CH_2), and 2 broad singlets (1-proton each) at δ6.83 and δ7.62 (imidazole ring protons). A picrate salt, m.p. 223–226° [lit. (1) 223–226°], and the dihydrochloride salt, m.p. 183.5-184° [lit. (1) 182–184°], were prepared and characterized by elemental analysis. Comparison of the material from the sponge *Ianthella* sp. with a synthetically prepared sample of 4(or 5)-[2-(dimethylamino)-

[1] A voucher specimen of sponge *Ianthella* sp. is maintained at A. H. Robins Co., Richmond, VA 23220

[2] Microanalyses were performed by Mr. Malcolm Stone of the A. H. Robins Co. Hypotensive activity was determined in the anesthetized dog preparation by Dr. Bernard V. Franko of the A. H. Robins Co.
[3] Floridin Co., Pittsburgh, Pa.
[4] The Johns-Manville Co., New York, N. Y.