CHROM. 3515

Combined chemical and isotopic detection of iodoamino acids in thin layer chromatography

EMRICH *et al*[1] as well as VOLPERT *et al.* have recently reported on the presence of radioactive impurities in preparations of labelled thyroid hormones used in physiological experiments. These authors claimed that it was necessary to check all shipments for radioactive contaminants before using them, in order to avoid false results. This procedure, however, does not exclude the presence of other—non radioactive—iodinated contaminants in the same sample. A combined chemical and isotopic investigation would seem to be advisable in order to obtain an accurate picture of the impurities present in a preparation; on the other hand, however, the relative lack of sensitivity of most of the techniques of chemical detection as compared with isotope detection precludes an adequate comparison of the two types of results.

The ferric chloride–(potassium)ferricyanide–arsenious acid (FFCA) reaction of GMELIN AND VIRTANEN[3] is sensitive enough to detect chemically minute quantities of iodinated compounds. Though originally meant to be applied in paper chromatography, it can also be used in thin-layer chromatography (TLC) as in a technique described elsewhere[4] and affords the possibility of detecting, chemically, microgram quantities of these substances, which produce bright blue spots on contact with the reagent. The pigment production depends on the reduction of the ferric and ferricyanide ions by the arsenious acid in the presence of catalytical quantities of iodide, but there are also other non-halogenated chemical structures which will give a colour response on contact with FFCA solution[5]. This fact does not diminish the effectiveness of the reagent in TLC, provided that the resolving power of the development systems used permit the previous satisfactory separation and identification of the colour producing compounds present in a mixture on the basis of their mobilities.

In order to assess the practicality of TLC combined with the GMELIN AND VIRTANEN reagent as a chemical method for detection of impurities in thyroid hormone batches, several samples of stable thyroxine and triiodothyronine, of different origins and analytical grades, were spotted in increasing quantities on inactive cellulose layers and run in a mobile phase of dilute acetic acid and acetone, suitable for the separation of different iodophenols[6]; the plates were stained with FFCA reagent.

After evaluating the results obtained, a comparative chemical and isotopic survey of the impurities in labelled thyroxine and triiodothyronine samples, using the above mentioned techniques in combination with radioautography, was carried out.

Material and methods

Stable triiodothyronine and thyroxine samples. Three samples (A,C,D) of 3,3′,5-triiodothyronine, one (B) of the sodium salt of 3,3′,5-triiodothyronine and three (E, F, G) of 3,3′,5,5′-tetraiodothyronine were employed*. The substances were

* Samples C and D were kindly donated by Farbwerke Hoechst AG., Germany, sample G by Deutsche Hoffmann-La Roche, sample A and sample F were purchased from Fluka AG., Switzerland, samples B and E from Serva-Entwicklungslabor, Germany.

dissolved at three different concentrations in a mixture of methanol–0.1 *N* NaOH (1:1, v/v) and spotted on the chromatographic layer in fixed volumes of 20 μl containing 0.05, 0.5 and 5 μg of free triiodothyronine or thyroxine.

Labelled triiodothyronine and thyroxine samples. A sample of ^{131}I-3,3',5-triiodothyronine and another of ^{131}I-3,3',5,5'-tetraiodothyronine of specific activity of 0.1 μC per 1 mg were employed*. The original solutions of 0.5 mg per 1 ml were diluted in order to obtain concentrations of 5, 0.5, 0.05, 0.005 and 0.0005 μg per 20 μl.

Thin-layer chromatography. The substances were run on 20 × 20 cm glass plates coated with cellulose powder with Gypsum MN 300 G, Macherey, Nagel & Co. (20 g MN 300 G + 120 ml distilled water for 5 plates). Chromatography was carried out in the ascending manner in glass tanks with acetone–0.5 *N* acetic acid (2:8, v/v) as mobile phase. The running time was approximately 1 1/2 hour. The plates were dried overnight at room temperature and then developed chemically.

Ferric chloride–(potassium)ferricyanide–arsenious acid reagent. The FFCA reagent is prepared immediately before use by mixing 5 parts of solutions A and B with 1 part of solution C and is then sprayed on the chromatograms.

Solution A: 2.7 g $FeCl_3 \cdot 6H_2O$ dissolved in 100 ml 2 *N* HCl.

Solution B: 3.5 g $K_3Fe(CN)_6$ dissolved in 100 ml distilled water.

Solution C: 5 g $NaAsO_2$ are dissolved in 30 ml *N* NaOH at 0° and mixed with vigorous stirring with 65 ml 2 *N* HCl.

Radioautography. The radioautography films (Du Pont Safety "Cronar" WG) were put in direct contact with the cellulose layer for 7 days and then developed.

*Results and discussion***

As Fig. 1 shows, even when the iodoamino acids are spotted in the lowest concentrations (0.05 μg per 20 μl) the corresponding spots are clearly visible, round

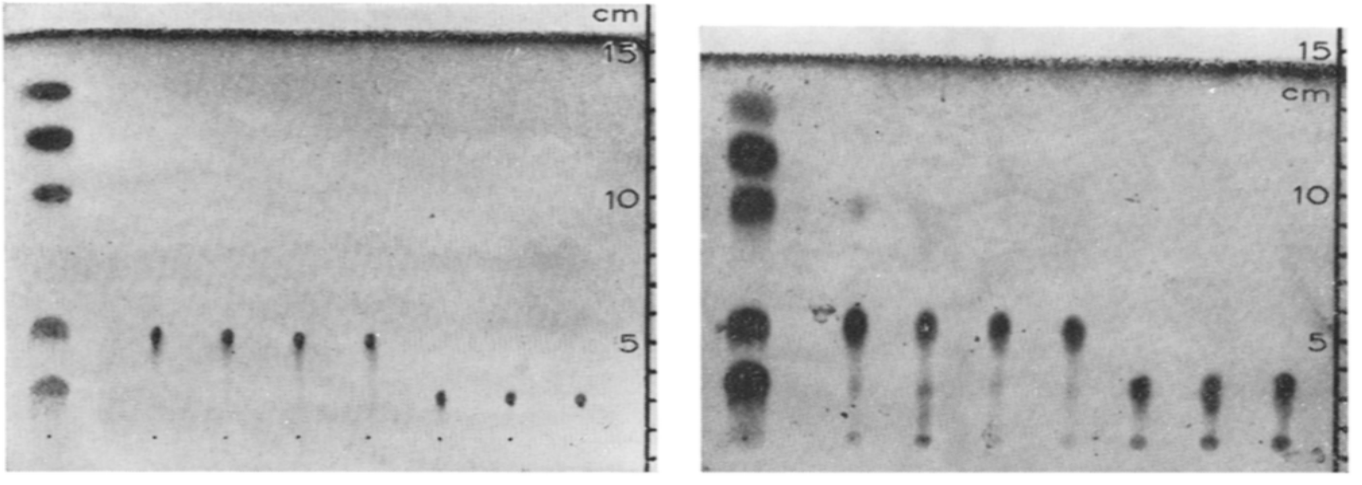


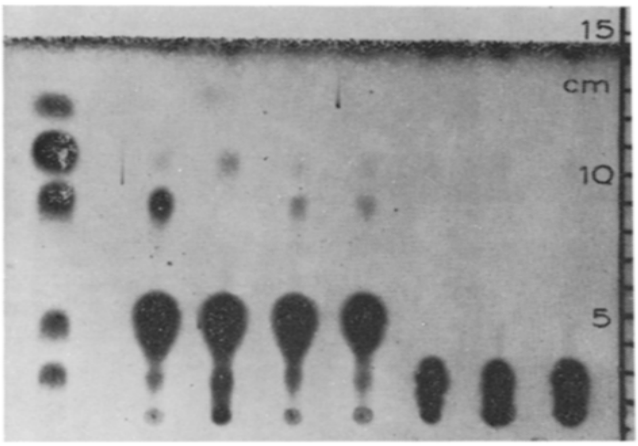
Fig. 1. Chromatograms of 0.05 μg of the tri- and tetra-iodothyronine samples stained with FFCA reagent. On the left side of the plate the chromatogram of the reference substances is visible (from top to bottom MIT, DIT, T_2, T_3 and T_4). From left to right are samples A, B, C, D and E, F, G.

Fig. 2. Chromatograms of 0.5 μg of the tri- and tetra-iodothyronine samples. The disposition of the samples, as well as of the reference substances, is the same as in Fig. 1.

* Kindly supplied by Farbwerke Hoechst AG., Germany.

** The following abbreviations are used; MIT and DIT for mono- and di-iodotyrosine; T_2, T_3 and T_4 for di-, tri- and tetra-iodothyronine.

and well defined, their mobilities corresponding strictly to those of the reference standards. If the spotted quantities are increased tenfold (Fig. 2), the four T_3 specimens become elongated and show a T_4 impurity embedded within the tails. Furthermore, the first T_3 sample shows a second spot located at the T_2 level. Considering the lowest limit of detection of the FFCA reagent and the fact that it failed to detect any impurity in the former chromatograms, the present quantity of reactive substance in these additional spots is estimated as > 0.01 and < 0.1 μg. On applying 5 μg of T_3 and T_4 to the layer (Fig. 3) the respective spots spread, thus becoming less well defined, and the impurities already described become more clearly visible. In addition, other new contaminants appear at the positions of T_2 and DIT. The T_4 samples show weak impurities corresponding to the DIT standard.



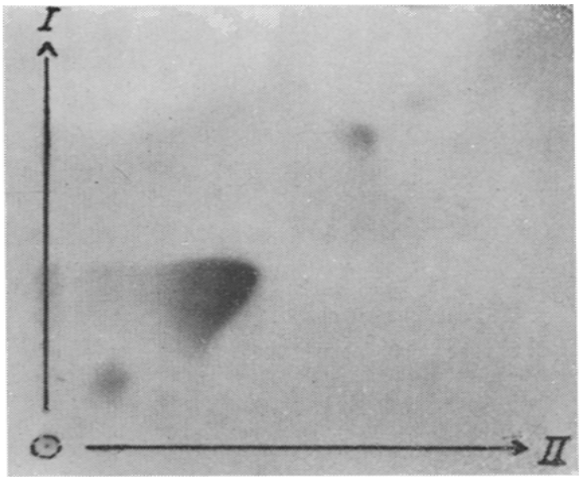


Fig. 3. Chromatograms of 5 μg of the tri- and tetra-iodothyronine samples. The disposition of the samples, as well as of the reference substances, is the same as in Fig. 1.

Fig. 4. Bidimensional chromatogram of 5 μg of sample A stained with FFCA reagent.

Instead of maintaining that these are impurities present in the samples, it could be argued that these additional spots are degradation products formed during the chromatographic process, in spite of the short running time and the mild conditions of the system. Bidimensional chromatograms of the samples in the same mobile phase failed, however, to reveal the appearance of new spots derived from the substances tested during the second run (Fig. 4). This indicates that the additional spots are in fact contaminants present in the samples and not chromatographic artifacts.

A minimum of 0.05 μg of tri- and tetra-iodothyronine become visible, as in the former case, when the chromatograms of the labelled substances are developed chemically (Fig. 5). In the case of the 5 μg specimen of T_3 the presence of an additional impurity becomes evident. The specific activity of the samples only permits the localization on the corresponding radioautogram of the plate (Fig. 6) at the 0.5 μg level of both compounds. The spots of 0.05 μg and the described impurity below the T_3 specimen appear only as light shadows or are not visible at all.

The co-ordination of methods, described above, for iodinated compounds is very suitable for the investigation of circulating thyroid hormones and derivatives, which produce a dissimilar pattern when revealed through chemical or isotopic

methods[7,8]. It is in current use in this laboratory for the elucidation of different problems of thyroid pathology and gives very good results in combination with an appropriate serum extraction[9,10]. Fig. 7 provides an example.

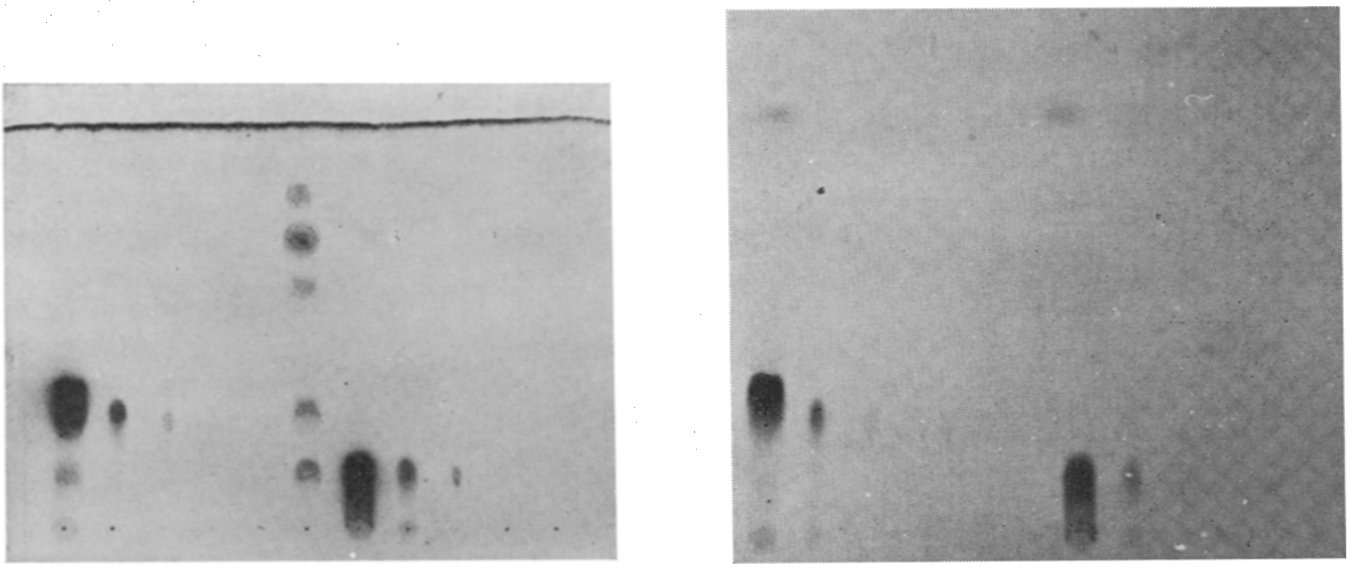


Fig. 5. Chromatograms of 5, 0.5, 0.05, 0.005 and 0.0005 μg of the labelled tri- and tetra-iodothyronine samples stained with FFCA reagent. On the left hand side, the T_3 samples (from left to right 5 to 0.0005 μg). On the right hand side, in the same order, the T_4 samples. The middle line is the chromatogram of the reference substances (from top to bottom MIT, DIT, T_2, T_3 and T_4).

Fig. 6. Radioautography of the plate shown in Fig. 5.

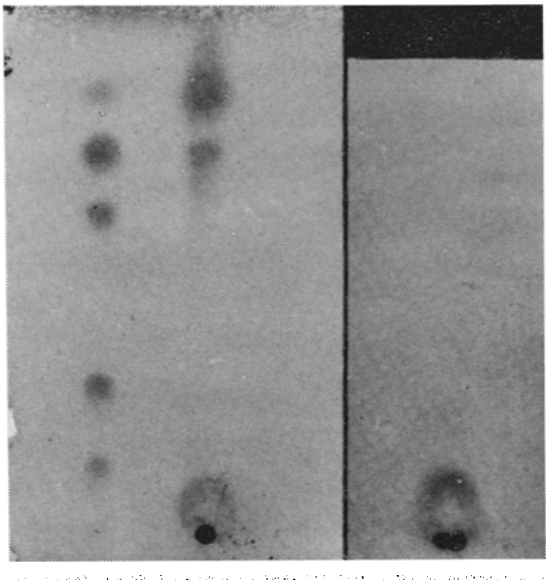


Fig. 7. Combined chemical and isotopic detection of thyroid hormones and iodinated derivatives in an extract of a sample of 5 ml serum from a patient who had received a 3 mC ^{131}I dose a week before. The chromatograms of the extract, stained with FFCA reagent show three spots which correspond from top to bottom to the MIT, DIT and T_4 reference substances (left). The radioautogram (right) shows only one spot, whose shape and position correspond to that of T_4 as revealed chemically.

Acknowledgements

The authors wish to express their thanks to Miss MARGRIT MARTIN, Miss GUDRUN BEDNARZ and Mr. FRED PRANGE for excellent technical assistance.

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Received March 18th, 1968

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CHROM. 3480

Separation of imidazoles in the histidine-loading test

The histidine-loading test, originally devised by LUHBY *et al.*[1], has been used clinically for about ten years now, mainly for the detection of deficiency of folic acid and/or vitamin B_{12}. Such a deficiency is shown by the appearance of urocanic acid[2] and/or formiminoglutamic acid (FIGLU) in the urine after an oral load of 15 g L-histidine monohydrochloride.

The screening of urine for these two metabolites was facilitated when ROBERTS AND MOHAMED[3] introduced a thin-layer chromatographic separation on cellulose, with an *n*-butanol–acetic acid–water solvent. In the same year, MIDDLETON[4], using two-dimensional paper chromatography, showed the importance of examining other histidine metabolites, especially other imidazoles.

From a technical point of view, there are two problems in the use of *n*-butanol–acetic acid–water as solvent. In the first place, as is well known, esterification occurs readily between the butanol and acetic acid, and the solvent gives a good separation of imidazoles only when freshly prepared. Secondly, especially in pregnancy cases where altered kidney function allows large quantities of histidine to appear in the urine, the chromatography consists of separating small quantities of metabolites in the presence of large amounts of histidine. This, in turn, gives rise to streaking of histidine, often reaching up to the spot of imidazolelactic acid.