A COMBUSTION METHOD FOR THE QUANTITATIVE ESTIMATION OF CHROMATOGRAPHIC SPOTS

I. DETERMINATION OF ORGANIC COMPOUNDS CONTAINING FLUORINE

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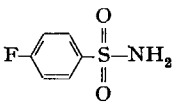
GENERAL INTRODUCTION

The quantitative determination of organic compounds that have been separated by paper chromatography or electrophoresis is generally carried out by means of a colour reaction. The colour intensity is usually measured after elution, but it can also be determined directly on the paper, the absorbance being measured under U.V. light. Most colour reactions are based on a reaction with a functional group. Few or no quantitative determinations of spots are, however, based on the determination of a single element present in the compound under investigation.

In the procedure published in 1955 by SCHÖNIGER[1] for the micro-determination of elements in organic compounds, a method has been designed that is very suitable for the quantitative determination of spots separated by chromatography or electrophoresis.

In the so-called SCHÖNIGER combustion, an organic compound is burned in an erlenmeyer flask containing oxygen and the combustion gases are absorbed in an appropriate liquid. In this way the organically bound elements are transformed quantitatively into the ion form. The combustion of for instance *p*-fluorosulphonamide with water, using peroxide as absorption liquid, can be summarised as follows:



$$C \longrightarrow CO_2$$

$$H \longrightarrow H_2O$$

$$N \longrightarrow N_2 \text{ and a small amount of } NO_3^-$$

$$S \longrightarrow SO_4^{2-}$$

$$F \longrightarrow F^-$$

The numerous publications and review articles[2–5] that have appeared since this method was first published, provide evidence of the great progress it has made in organic micro-chemistry.

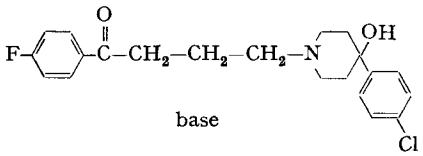
For a description of the (very inexpensive) apparatus and the procedure, the reader is referred to the original paper[1]. In this procedure an aliquot of the substance is weighed so as to obtain ± 0.05 mequiv. of the element to be determined. This

amount is, however, much larger than those obtained by paper chromatography or electrophoresis, as the spots often contain not more than 0.1 μequiv. of the element to be determined. The existing micro-methods for the determination of the following organically bound elements in the oxygen flask, C, Cl, Br, I, F, S, P, As, B, Zn, Hg, Cd and Se[6], should therefore be modified and scaled downward (sub-micro scale), or new methods should be designed.

SUB-MICRO DETERMINATION OF ORGANICALLY BOUND FLUORINE

Among many butyrophenone derivatives synthetized in this laboratory, a new compound has been prepared with potent neuroleptic properties and bearing some resemblance to the phenothiazine derivatives. This compound, called Haloperidol, is highly active in very small concentrations[7].

Structure



base

Laboratory name

R 1625

The numerous experiments, the main object of which was to gain a better knowledge of the mode of action of the drug, were also made with the aim of studying the *in vivo* and *in vitro* metabolism of Haloperidol in rats.

Various reactions were performed to characterise the compound after extraction from biological material. A variety of ketone reagents[8–10] were used, all of which gave negative results. This may perhaps be attributed to an interaction between the negative carbonyl group and the positive nitrogen atom in the heterocyclic nucleus. Reactions for the heterocyclic nitrogen atom proved to be qualitative but not quantitative[11]. The possibility was, therefore, considered of carrying out a chlorine or fluorine determination after chromatographic separation of the compound. Since animal tissues contain much more chlorine than fluorine, a fluorine determination will be more specific. Furthermore, no basic organic fluorine compounds (which would also be extracted in the method used) seem to be present in rat tissue, urine or faeces, while the inorganic salts containing fluorine, such as CaF_2 and NaF are not determined. This is important, in view of the fact that the dose of Haloperidol under investigation is only 40 γ per rat, which corresponds to 2 γ F/rat. If NaF and CaF_2 were also determined, the blank value would be much too high.

The method used, which is described below, can be summarised as follows:

1. Extraction of the substance from biological material.
2. Concentration of the extract.
3. Chromatography.
4. Visualisation of the spots.
5. Determination of the fluorine content of the spots.

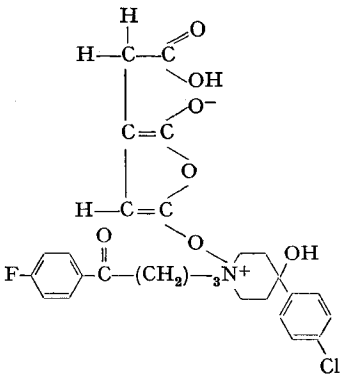
Methods

Extraction from biological material (urine, faeces, tissue). The sample is adjusted to pH 12 with 10 *N* NaOH and shaken with 5 vol. of ethyl ether. The ether layer is evaporated to ± 5 ml *in vacuo* and the base extracted with 2 vol. (10 ml) 0.1 *N* HCl.

Concentration of the extract. After extraction of a substance, either in water or in organic solvent, the total volume can be reduced to a very small known amount (*e.g.* from 10 ml to 50 μl) by the very interesting method described by CLARKE AND HAWKINS[12]. This method allows the determination of the total amount of extracted substance on a single chromatogram.

Chromatography. Experiments were made with buffered paper and the solvent described by CURRY AND POWELL[11]. The R_F value in this solvent (the upper layer of 50 ml butanol, 50 ml water, 1 g citric acid) was, however, between 0.95 and 1.0. The buffer paper was retained, but another solvent was used: methanol–amyl alcohol–benzene–water, 40:20:40:5. To avoid interference of the fluorine determination, the solvents should be free of traces of heavy metals (Dithizon test).

Visualisation of the spots. The spots are visualised under an U.V. lamp. It is, however, possible to spray with a reagent containing elements that after combustion do not interfere with the fluorine determination. A modified DRAGENDORFF reagent[13] containing bismuth cannot be used because bismuth interferes with the fluorine determination; a reaction with, for example, *cis*-aconitic acid in acetic anhydride[14,15] is, however, possible. In this reaction the following violet coloured complex is probably formed:



Buffered paper may not be used in this case, as alkali salts of organic acids give a positive reaction[15]. This reaction will be studied further; a sensitivity of 0.1 γ Haloperidol was obtained in a preliminary investigation.

Determination of fluorine. The decrease in fluorescence of the aluminium–morin complex induced by increased quantities of fluoride ion forms the basis of a standard method for fluoride determinations. This procedure was used by BOUMAN[16] for the determination of fluorine in the range of 0.005 γ – 0.08 γ. We have found, however, that the complex formed is very unstable and that the measurements vary frequently.

When 25 γ of fluorine are present after chromatography, a titration can be performed in a volume of 5–10 ml with a relative error of $\pm$ 2 %. This method is a modification of a micro-titration[5] in which thorium nitrate is used as the titrant and alizarinsulphonate–methylene blue as the indicator.

For smaller amounts (0.1–2 γ F) a slightly modified method of SINGER AND ARMSTRONG[17] was found satisfactory: the decoloration of the zirconium–Eriochrome Cyanine R complex is proportional to the fluoride concentration.

Apparatus

A modified SCHÖNIGER Erlenmeyer flask of 150 ml with a B 24 stopper with platinum wire and a small valve fitted at the bottom of the Erlenmeyer, as shown in Fig. 1.

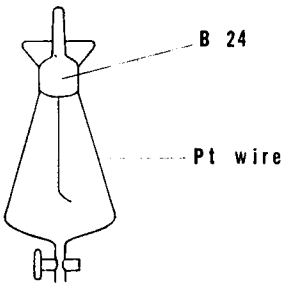


Fig. 1. Modified SCHÖNIGER oxygen flask.

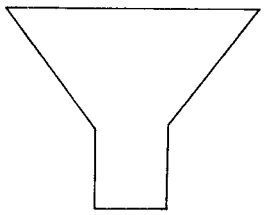


Fig. 2. Submicro titration vessel. Full size. The liquid in the upper part must be evaporated before the titration is carried out in the cylindrical part.

Colorimeter tubes, size 10 ml (borosilicate glass).
Titration vessel for submicro titration as shown in Fig. 2.
Microburette: 0.3 ml (Prolabo).
Apparatus for drying strips of filter paper (Fig. 3).

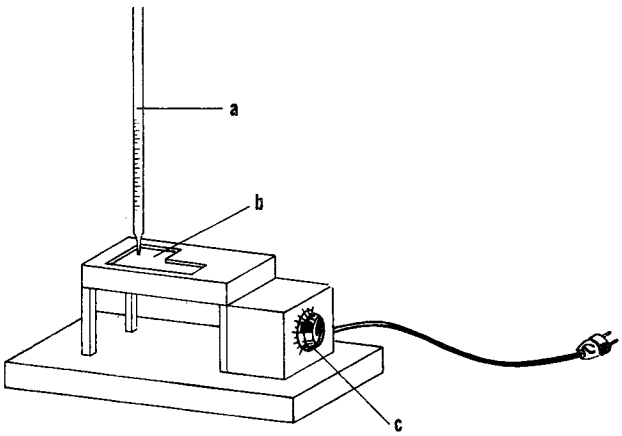


Fig. 3. Apparatus for drying strips of filter paper. (a) Pipette of the apparatus of CLARKE AND HAWKINS[12]; (b) a spot cut out from a chromatogram; (c) variable resistance.

Eppendorf-Colorimeter with mercury-lamp, 546 mμ filter and cuvettes of 4 cm light path.

Reagents

Redistilled water: Laboratory distilled water is deionized by passing it through a mixed-bed resin (Elgastat) until the resistance reaches 800,000 Ω/cm. The water is stored in a polyethylene bottle.

Solution a: 210.7 mg of Eriochrome Cyanine R (Merck) dissolved and diluted to 100 ml with water and stored in a polyethylene bottle.

Solution b: 30.5 mg zirconylchloride octahydrate (Merck) dissolved in 161.4 ml concentrated hydrochloric acid and diluted to 200 ml with water (polyethylene bottle).

Reagent solution: 1 vol. of solution a added to 2 vol. of solution b. This solution is prepared fresh daily.

Fluoride stock solution: 22.1 mg p.a. sodium fluoride (Merck) dissolved in 100 ml of water (polyethylene bottle).

Standard solution: 2 ml of the stock solution diluted to 200 ml with water (1 γ F/ml).

Calibration curve with sodium fluoride

In a series of 6 colorimeter tubes (10 ml), 0, 0.4, 0.8, 1.2, 1.6 and 2.0 ml of the standard fluoride solution (1 γ/ml) are placed; 0.5 ml of the reagent is added and the solution is

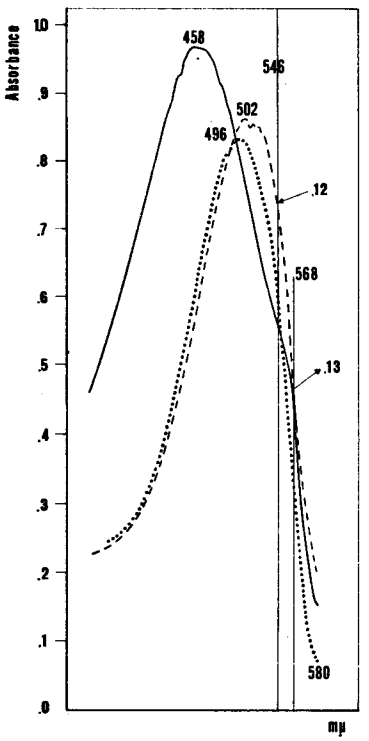


Fig. 4. Absorbances of: the Eriochrome Cyanine R solution, with a peak at 458 mμ (——); the reagent complex, peak at 502 mμ (– – – –); the reagent complex + 2 γ fluoride, peak at 496 mμ (. . . .). The absorbances are measured *versus* water in a Beckman DK_2 apparatus with cuvettes of 1 cm.

then diluted to 10 ml with water. The tubes are shaken and the extinctions measured 1 h later. The solutions containing fluoride are used as blank solutions. The extinctions are measured at 546 mμ in a cuvette of 4 cm. Fig. 4 was drawn in order to find a wavelength at which there is sufficient difference between the blank and the sample.

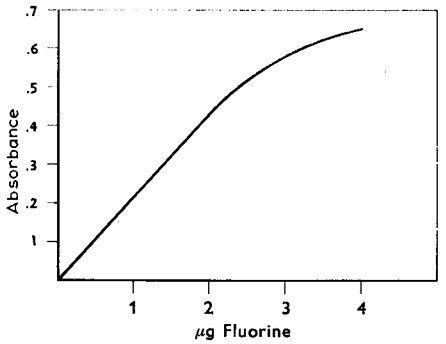


Fig. 5. Calibration curve of a sodium fluoride solution. Absorbances of the zirconium–Eriochrome Cyanine R complex with 0–2 γ fluoride in a total volume of 10 ml.

As can be seen in Fig. 4, the region between 580 mμ and 540 mμ is very suitable for measurements. SINGER AND ARMSTRONG made the measurements at 568 mμ in a Coleman spectrophotometer. In our method, the measurements are performed at 546 mμ. Both measurements are almost equivalent.

Fig. 5 shows that the relation between fluoride concentration and extinction is linear within the range of 0–2 γ F.

Calibration curve after combustion of an organic substance containing fluorine

A solution containing 1 mg Haloperidol "purissimum" per ml of 0.1 *M* tartaric acid is introduced into a microburette (0.3 ml); 0, 8, 16, 24, 32 and 40 μl of this solution are then placed on buffered paper. The spots are cut out under an U.V. lamp as indicated in Fig. 6. The strips are fixed to the platinum wire of the B 24 stopper (Fig. 1).

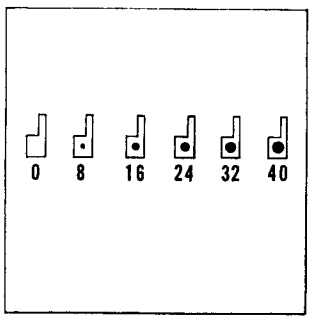


Fig. 6. Six spots are placed on a Whatman No. 1 paper and dried; their outlines are traced with a pencil under U.V. light. The spots are cut out along the pencil line.

The combustion flask (Fig. 1) is filled with 2 ml water and flushed with a strong flow of oxygen for 30 sec; the strip is ignited and the flask closed. After absorption of the

combustion gases, the neck around the stopper of the combustion flask and the platinum wire are rinsed with 2 ml water. The valve is opened and the absorption liquid collected in a colorimeter tube. The flask is again rinsed with 5 ml water and this is added to the first 4 ml, 0.5 ml reagent is added, the total volume diluted to 10 ml and the extinction measured after 1 h as described above for the NaF solution. Fig. 7 indicates that after combustion the same extinctions as in Fig. 5 are found for the same amounts of fluorine. The deviations are however 20–30 %.

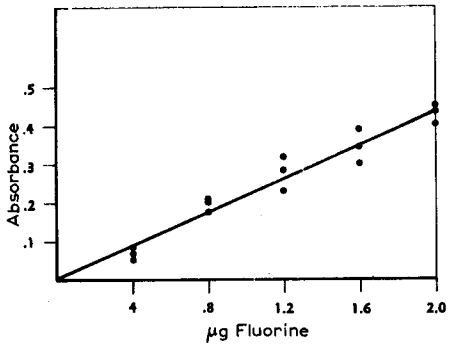


Fig. 7. Calibration curve after combustion of various amounts of an organic substance (Haloperidol) containing 0–2 γ fluorine. Absorbances of the zirconium–Eriochrome Cyanine R complex.

Calibration curve after chromatography

The calibration curve was drawn using the same method as for Fig. 7, except that the 6 spots were placed at one end of the paper. After ascending chromatography the papers are dried at 65° for 30 min. The spots with R_F values between 0.75 and 0.80 that are visible under an U.V. lamp are cut out. The procedure is further carried out as described for the calibration curve of Fig. 7.

RESULTS

From Fig. 8 it can be seen that after chromatography the extinctions for the same amounts of fluorine have decreased, probably owing to loss during the procedure.

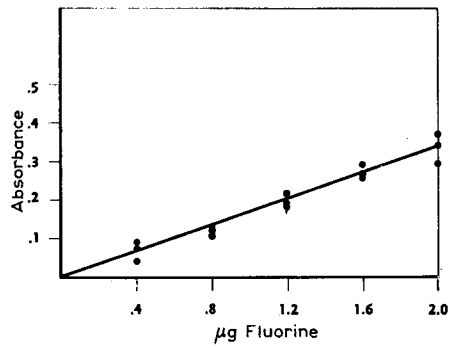


Fig. 8. Calibration curve after chromatography and combustion of various amounts of an organic substance (Haloperidol) containing 0–2 γ fluorine. Absorbances of the zirconium–Eriochrome Cyanine R complex.

The average values of the results, calculated on the "best fitting line", varied between 85 % and 110 %.

DISCUSSION

When the test solution contains only one fluorine compound, it is not necessary to separate the compound by chromatography; the compound to be determined can be placed on the strip after evaporation of the solvent. The extract is then introduced into the pipette described by CLARKE[12] and dripped onto the strip which is placed on an adjustable heating disk (Fig. 3). A volatile solvent (alcohol, ether) is preferable because the evaporation is more rapid.

Aliquots of Haloperidol up to 1 mg (50 γ F) were added to 50 ml rat urine and part of the ether extract was added dropwise onto the strip. The recovery was 90 %.

SINGER AND ARMSTRONG[17] studied the influence of sulphate and phosphate on the fluorine determination and observed that less than 30 γ sulphate did not interfere and that 5 γ phosphate gave the same extinctions as 0.1 γ fluorine. The new method of determining fluorine described by BELCHER *et al.*[18,19] (a direct colorimetric determination) might give better results. This will be investigated in the future.

The method described above is not limited to fluorine. Determinations of sulphur-containing amino acids should be possible by means of a submicro-sulphate determination and phosphorus in a compound by means of a phosphate determination. This will also be investigated in the future. In brief, any substance containing an element that can be determined with the oxygen flask lends itself to this technique. Carbon determinations can be made by eluting the substance in a suitable solvent, transferring it dropwise to a combustion boat and igniting it electrically. A combustion flask similar to those already described[20–22], but containing a valve at the bottom of the flask, should be designed.

Substances separated on chromatoplates can be determined as follows: the spot with the layer is cut out and placed in a centrifuge tube, an appropriate solvent is added and the solution centrifuged. The supernatant is pipetted onto a strip (Fig. 3).

Compounds containing no characteristic SCHÖNIGER element (*i.e.* compounds containing only C, H, N and O) can be determined by allowing them to react with a compound which does have a characteristic element and then making a chromatogram of the complex. Adrenaline and noradrenaline (C, H, N, O) form stable boric acid complexes[23]. The percentage of boron can be determined after chromatographic separation by a recently described fluorimetric method[24].

Preference should be given to heavy metal complexes providing they do not react with the platinum wire during combustion, because there are very accurate methods for such determinations.

Much depends on the accuracy with which the element can be determined on the submicro-scale. If the accuracy is greater than that of a given colour reaction for the spot or if this reaction is not sensitive enough, it is certainly worth while trying the above described method for the quantitative estimation of spots separated by paper chromatography or by paper electrophoresis.

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SUMMARY

A method for the quantitative estimation of chromatographic spots of fluorine-containing organic compounds has been developed. The procedure is based upon the colorimetric determination of the fluoride ion (range 0–2 γ F) formed after combustion of the fluorine-containing spot in a "SCHÖNIGER oxygen flask". The possibility of using this method for the determination of other elements is discussed.

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