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3,817,706

FLUORESCENCE QUANTITATIVE THIN LAYER
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No Drawing, Filed Oct. 16, 1972, Ser. No. 297,639

Int. Cl. G01n 21/22, 31/08

U.S. Cl. 23—230 B

4 Claims

ABSTRACT OF THE DISCLOSURE

Method of inducing fluorescent chromophores into a variety of organic substrate molecules. Once introduced into the molecule to be analyzed, the fluorescent chromophore permits rapid and accurate quantitative analysis of the substrate precursor by standard fluorescent spectrophotometric techniques. The process of introducing the chromophore involves impregnating the adsorbent material with ammonium sulfate, simultaneously developing the unknown with known standards, exposing the developed plate to *tert*-butyl hypochlorite, and heating the chromatogram. Amino acids, fatty acids, triglycerides, sugars, steroids and prostaglandins are analyzed by this technique in microgram quantities.

The present invention involves a method of fluorescent chromophoric labeling of a wide variety of organic molecules on thin layer plates to enable quantitative analysis of the organic precursors. In this method, the adsorbent of the thin layer plate is impregnated with 0.25 to 20% of ammonium sulfate, the plate is spotted with knowns and unknowns, and developed. The developed plate is exposed to *tert*-butyl hypochlorite either before or during heating. The intensity of fluorescent light is measured by standard spectrophotometric techniques.

Thin layer chromatography is a simple and inexpensive method of separating complex mixtures of organic molecules and of qualitatively identifying the components of the mixture. Numerous methods of quantitatively estimating the amount of a given component are also available.

These methods include adsorbence, reflectance transmission, and fluorescence spectroscopy.

In fluorescence spectroscopy the molecules on a thin layer plate are irradiated with light. The incident light promotes an electron of the absorbing chromophore from the ground state to an excited singlet state. As the excited electron relaxes from the singlet state back to the ground state, light of a characteristic spectrum is emitted. The spectrum of emitted light is a function of the absorbing chromophore and the molecule to which the chromophore is attached. Molecules which are without fluorescent chromophores must have these chromophores introduced for fluorescent techniques to be applicable. Chromophore labeling is often accomplished by preparing fluorescent functional group derivatives of the molecules under consideration. For example, biogenic amines are specifically labeled with fluorescent chromophores by formaldehyde vapor, L. S. Van Orsen, *Biochemical Pharmacology*, 19, 1105, (1970). The condensation product between the amine and formaldehyde is the fluorescent chromophore. The specificity of reagent requirement necessitates a large number of reagents for quantitative analysis of compounds of various structure. There is also present the problem of applying the reagent to the chromatographic plate in a uniform manner. For example, spraying is the most common technique of reagent application and unevenness of spraying introduces a substantial error into quantitative analysis.

Sulfuric acid is a general purpose chromophore inducing reagent. The application problem has been overcome

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by impregnating the adsorbent material with ammonium hydrogen sulfate and releasing the sulfuric acid after development of the plate by heating (Wortman *et al.*, *J. Chromatogr.*, 66, 172, (1972)). Although fluorescent labeling by sulfuric acid released from ammonium hydrogen sulfate is broader in scope than specific functional group fluorescent labeling, it is applicable to a much narrower group of compounds than the methods of the present invention. For example, fluorescence from oleic acid is barely visible when the chromophores are introduced by the method of Wortman *et al.* (*supra*). Visible emissions of good intensity are obtained by ammonium sulfate-*tert*-butyl hypochlorite heat method of the present invention. This is an indication of the greater sensitivity of the present method in quantitative analysis.

Vapor phase chlorination with *tert*-butyl hypochlorite has been reported as a technique for specifically introducing chromophoric groups into N—H containing compounds, P. S. Cammarata *et al.*, *J. Bio. Chem.*, 273, 1619 (1961). While vapor phase chlorination has the necessary feature for quantitative analysis of uniform application of reagent, it is not applicable to a broad spectrum of organic compounds.

The present invention provides a method of chromophoric labeling of a wide variety of organic compounds so that these compounds are amenable to quantitative fluorescent analysis. It is the combination of steps of vapor phase chlorination with *tert*-butyl hypochlorite and heating plates containing ammonium sulfate dispersed in the adsorbent which broadens the scope of compounds susceptible to quantitative thin layer analysis. The order in which the steps are conducted is not important. Table 1 illustrates the scope and sensitivity of the combination of the above steps in introducing fluorescent chromophores as compared to either step alone.

TABLE 1

Column A contains the results of plates treated with *tert*-butyl hypochlorite alone; Column B contains the results of plates treated with ammonium sulfate alone, and Column C contains the results of treating the plates in accordance with the present invention. The brightness represents the total visible light emitted by fluorescence when the excitation wave length is 365 nm.

	A	B	C
	t-Butyl hypo- chlorite	Ammo- nium sulfate	Comb- ination
1 Cholesterol n-propionate.....	++*	+	+++++
2 Cholesterol.....	+	+	+++
3 Androstane-3,17-dione.....	±	±	+++
4 Androstene-3,17-dione.....	++	+	+++
5 Δ ^{1,4} androstene-3,17 dione.....	+	±	+++
6 Testosterone.....	++	+	+++
7 Xanthanoic acid.....	+++	+	+++++
8 D-glucose.....	—	++++	+++++
9 D-mannose.....	—	+++	++++
10 Oleic acid.....	±	+	+++
11 Stearic acid.....	±	+	+++
12 Phenobarbital.....	—	±	+
13 Methyl phenylalanine.....	—	±	+
14 Morphine.....	±	—	++
15 Cocaine-HCl.....	±	—	++
16 Dopamine.....	+	+	+++

*The scale of fluorescent brightness is judged by eye from a photograph of the emitted light and the scale is: —=not visible; ±=barely visible; + = low intensity; ++ = intermediate intensity; +++ = medium intensity; ++++ = bright; +++++ = extremely bright.

The present method of fluorescent chromophoric labeling is particularly suitable to quantitative analysis in that the ammonium sulfate and *tert*-butyl hypochlorite are applied to the chromatographic plate in a uniform manner, the former being dispersed in the adsorbent through solution and the latter being applied in the vapor phase. Another important feature of the present invention, in regard to quantitative analysis, is that the fluorescent response is linear over a long period of time (3 days). Therefore, it is not necessary to conduct the fluorescent analysis immediately after development of

the plate. The present invention is particularly distinct from other methods of fluorescent chromophore labeling in that it provides for increased sensitivity in the quantitative analysis of a broader range of compounds than heretofore known methods. The classes of organic compounds to which the method of the present invention is applicable is indicated in Table 1 and details of particular analysis are set out in the examples. The compounds listed in Table 1 and in the examples are intended to indicate the classes of compounds which can be analyzed by methods of this invention and are not to be construed as compounds to which these methods of analysis are limited.

An adsorbent, such as alumina, silicic acid or silica gel may be used in the preparation of the chromatographic media. A slurry is prepared from a solution of 0.25 to 20 parts of ammonium sulfate in 60 parts by volume of water and 30 parts of adsorbent (silica gel). The adsorbent slurry is spread on clean chromatographic plates as a layer 250 microns in thickness. The plates are allowed to dry for 24 hours at room temperature and standards and unknowns are applied to the plates in uniform volume. The plates are developed in vapor saturated chambers in solvent systems such that the R_f value is between 0.3 to 0.75. The adsorbent, film thickness, type and size of plates, and solvent system are variable and are not considered essential elements of the present invention. After development the plates are dried in a stream of nitrogen for 30 minutes and placed in a chamber saturated with *tert*-butyl hypochlorite vapors and immediately placed in an oven at 120–200° C. for 15 minutes. After cooling, the plates are scanned with a Zeiss PMQ-2 spectrophotometer equipped for thin layer chromatography scanning. The excitation wave length is 365 nm. and the optimum fluorescent emission is determined and observed. The latter varies with the substrate being analyzed. The data is recorded as peak height of the emission maxima as well as the area under the emission curve resulting from scanning the emission from the thin layer spot at a specific wave length.

Alternatively, the plate containing ammonium sulfate in the adsorbent is heated first at 120–200° C. for 15–30 minutes and then exposed at the same temperature to *tert*-butyl hypochlorite vapors. The latter is accomplished by saturating an undeveloped plate with *tert*-butyl hypochlorite and placing the undeveloped plate in the oven with the developed plate so that vapors of *tert*-butyl hypochlorite flow on to the plate to be developed. Both procedures produce essentially the same results.

A chromatographic plate in which silica gel G support contains 5% ammonium sulfate is spotted with 1.05, 2.06, 3.06, 4.07, and 5.07 $\mu\text{g.}$ of androstane-3,17-dione. The plate is developed in an ethyl acetate-benzene (30–70) v./v. solvent system. The R_f (ratio of the distance migrated by compound to distance migrated by solvent front) is 0.52 for androstane-3,17-dione in this solvent system. The plate is dried and exposed to *tert*-butyl hypochlorite vapors for 30 minutes. Then the plate is heated at 175° C. for 30 minutes. The spots are sequentially irradiated with light of 365 nm. and the fluorescent emission at 600 nm. is recorded. A plot of the peak height of the 600 nm. emission versus the $\mu\text{g.}$ of androstane-3,17-dione present provides a series of points which lie on a straight line. A similar plot using the area under the scanning curve versus concentration produces a series of points which lie on a straight line. The unknowns are estimated from the standard line.

Reproducibility is determined with cholesterol *n*-propionate on silica-gel containing 5% ammonium sulfate. The plate is spotted with three 1.5 $\mu\text{g.}$ portions of cholesterol *n*-propionate and developed with 100% benzene. The developed plates are dried, exposed to *tert*-butyl hypochlorite, and heated at 175° C. for 30 minutes. Fluorescence is induced by light of 365 nm. and observed at 600 nm. The percent relative standard deviation of the area under the emission curve is ± 2.7 . When cholesterol *n*-propionate

spots are fluorescent labeled with sulfuric acid spraying, the percent relative standard deviation of the area under the 600 nm. emission curve is ± 11.3 .

To determine the instrumental reproducibility and the effect of repeated scans on the stability of the fluorescence during the new procedure, the spots are scanned 5 times. The percent relative standard deviation of the area under the curve is $\pm 1.5\%$ and the percent relative standard deviation of the peak height is $\pm 0.88\%$. When the sample is developed with sulfuric acid spraying, the percent relative standard deviation of the area under the curve is $\pm 2.11\%$ and the percent relative standard deviation of the peak height is $\pm 1.4\%$.

EXAMPLE 1

1,2,3,4,5 $\mu\text{g.}$ of methyl phenylalanine are developed on silica gel G, containing 5% ammonium sulfate, thin layer plates, which are 250 microns thick, with (64–30–4–2) v./v. of chloroform-methanol-water-acetic acid. The R_f value is 0.36. The developed plates are dried and exposed to *tert*-butyl hypochlorite vapor for 30 minutes. The plates are then heated for 30 minutes at 175° C. The fluorescence is measured with a Zeiss PMQ-2 spectrophotometer using an excitation wave length of 365 nm. and observing the intensity of the fluorescent light at 580 nm. A plot of $\mu\text{g.}$ of standard versus the peak height of 580 nm. fluorescent scan past the given spot on the plate or the area under the curve of the fluorescent scan is essentially linear and unknowns developed on the same plate at the same time and within the range of standards are determined from this plot.

EXAMPLE 2

1,2,3,4,5 $\mu\text{g.}$ of androstane-3,17-dione are developed on silica gel G, containing 5% ammonium sulfate, thin layer plates, which are 250 microns thick, with (30–70) v./v. of ethyl acetate-benzene. The R_f value is 0.52. The developed plates are dried and exposed to *tert*-butyl hypochlorite vapor for 30 minutes. The plates are then heated for 30 minutes at 175° C. The fluorescence is measured with a Zeiss PMQ-2 spectrophotometer using an excitation wave length of 365 nm. and observing the intensity of the fluorescent light at 600 nm. A plot of $\mu\text{g.}$ of standard versus the peak height of 600 nm. fluorescent scan past the given spot on the plate or the area under the curve of the fluorescent scan is essentially linear and unknowns developed on the same plates at the same time and within the range of standards are determined from this plot.

EXAMPLE 3

1.6, 1.8, 2.0 $\mu\text{g.}$ of a prostaglandin PGE_1 are developed on silica gel G, containing 5% ammonium sulfate, thin layer plates, which are 250 microns thick, with (110–100–30–20) v./v. of ethyl acetate-water-2,2,4-trimethylpentane-glacial acetic acid (top layer). The R_f value is 0.39. The developed plates are dried and exposed to *tert*-butyl hypochlorite vapor for 30 minutes. The plates are then heated for 30 minutes at 160° C. The fluorescence is measured with a Zeiss PMQ-2 spectrophotometer using an excitation wave length of 365 nm. and observing the intensity of the fluorescent light at 600 nm. A plot of $\mu\text{g.}$ of standard versus the peak height of 600 nm. fluorescent scan past the given spot on the plate or the area under the curve of the fluorescent scan is essentially linear and unknowns developed on the same plate at the same time and within the range of standards are determined from this plot.

EXAMPLE 4

1,2,3,4,5 $\mu\text{g.}$ of oleic acid are developed on silica gel G, containing 5% ammonium sulfate, thin layer plates which are 250 microns thick with (25–1–74) v./v. of ethyl acetate-acetic acid-heptane. The R_f value is 0.41. The developed plates are dried and exposed to *tert*-butyl hydrochlorite vapor for 30 minutes. The plates are then

heated for 30 minutes at 160° C. The fluorescence is measured with a Zeiss PMQ-2 spectrophotometer using an excitation wave length of 365 nm. and observing the intensity of the fluorescent light at 580 nm. A plot of $\mu\text{g.}$ of standard versus the peak height of 580 nm. fluorescent scan past the given spot on the plate or the area under the curve of the fluorescent scan is essentially linear and unknowns developed on the same plate at the same time and within the range of standards are determined from this plot.

EXAMPLE 3

4,6,8,10 $\mu\text{g.}$ of triolein are developed on silica gel G, containing 5% ammonium sulfate, thin layer plates, which are 250 microns thick, with (7-92-1) v./v. of ethyl acetate-heptane glacial acetic acid. The R_f value is 0.46. The developed plates are dried and exposed to *tert*-butyl hypochlorite vapor for 30 minutes. The plates are then heated for 30 minutes at 180° C. The fluorescence is measured with a Zeiss PMQ-2 spectrophotometer using an excitation wave length of 365 nm. and observing the intensity of the fluorescent light at 580 nm. A plot of $\mu\text{g.}$ of standard versus the peak height of 580 nm. fluorescent scan past the given spot on the plate or the area under the curve of the fluorescent scan is essentially linear and unknowns developed on the same plate at the same time and within the range of standards are determined from this plot.

EXAMPLE 6

1,2,3,4,5 $\mu\text{g.}$ of glucose are developed on silica gel G, containing 5% ammonium sulfate, thin layer plates, which have an adsorbent layer 250 microns thick, with (90-5-5) v./v. of ethanol-acetic acid-water. The R_f value is 0.53. The developed plates are heated in an oven at 170° C. for 15 minutes and then another silica gel plate which contains 3 mls. of *tert*-butyl hypochlorite is placed in the oven so that the *tert*-butyl hypochlorite vapors come in contact with the developed plate. Heating is continued for 15 minutes and the plates are cooled. The fluorescence is measured with a Zeiss PMQ-2 spectrophotometer using an excitation wave length of 365 nm. and observing the intensity of the fluorescent light at 440 nm. A plot of $\mu\text{g.}$ of standard versus the peak height of 440 nm. fluorescent scan past the given spot on the plate or the area under the curve of the fluorescent scan is essentially linear and unknowns developed on the same plate at the same time and within the range of standards are determined from this plot.

EXAMPLE 7

0.01, 0.02, 0.04, 0.06, 0.08 and 0.10 $\mu\text{g.}$ of xanthanoic acid are developed on silica gel G. containing 5% ammonium sulfate, thin layer plates, which have an adsorbent layer 250 microns thick, with (20-1-79) v./v. of ethyl acetate glacial acetic acid-benzene. The R_f value is 0.35. The developed plates are heated in an oven at 170° C. for 15 minutes and then another silica gel plate which contains 3 mls. of *tert*-butyl hypochlorite is placed in the oven so that the *tert*-butyl hypochlorite vapors come in contact with the developed plate. Heating is continued for 15 minutes and the plates are cooled. The fluorescence is measured with a Zeiss PMQ-2 spectrophotometer using an excitation wave length of 365 nm. and observing the intensity of the fluorescent light at 440 nm. or 500 nm. A plot of $\mu\text{g.}$ of standard versus the peak height measured at 440 nm. or 500 nm. scan is essentially linear and unknowns developed on the same plate at the same time

and within the range of standards are determined from this plot.

EXAMPLE 8

0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 $\mu\text{g.}$ of cholesterol *n*-propionate are developed on silica gel G, containing 5% ammonium sulfate, thin layer plates, which have an adsorbent layer 250 microns thick, with 100% benzene. The R_f value is 0.50. The developed plates are heated in an oven at 170° C. for 15 minutes and then another silica gel plate which contains 3 mls. of *tert*-butyl hypochlorite is placed in the oven so that the *tert*-butyl hypochlorite vapors come in contact with the developed plate. Heating is continued for 15 minutes and the plates are cooled. The fluorescence is measured with a Zeiss PMQ-2 spectrophotometer using an excitation wave length of 365 nm. and observing the intensity of the fluorescent light at 600 nm. A plot of $\mu\text{g.}$ of standard versus the peak height of 600 nm. fluorescent scan past the given spot on the plate or the area under the curve of the fluorescent scan is essentially linear and unknowns developed on the same plate at the same time and within the range of standards are determined from this plot.

What is claimed is:

1. A process for quantitative thin layer chromatographic analysis of steroids, prostaglandins, fatty acids, triglycerides, amino acids and sugars comprising:

- (a) preparing chromatographic plates from adsorbent which is impregnated with ammonium sulfate;
- (b) developing standards and unknowns on said chromatographic plates;
- (c) contacting the chromatographic plates with *tert*-butyl hypochlorite vapors;
- (d) heating the chromatographic plates; and
- (e) analyzing the standards and unknowns by fluorescent spectrophotometric means.

2. A process as in claim 1, wherein the *tert*-butyl hypochlorite vapors are contacted with the chromatographic plates during heating.

3. A process as in claim 1, wherein the adsorbent of the chromatographic plates is impregnated with 0.25 to 20% ammonium sulfate and the heating is conducted at 100° to 200° C.

4. A process as in claim 1, wherein the adsorbent of the chromatographic plates is impregnated with 5% ammonium sulfate, the *tert*-butyl hypochlorite vapors are contacted with the chromatographic plates for 30 minutes, and the chromatographic plates are then heated at 150-180° C. for 30 minutes.

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U.S. Cl. X.R.

23-253 TP; 73-61.1 C; 250-302, 459