ortho-PHTHALALDEHYDE AS A SPRAY REAGENT FOR THIN LAYER CHROMATOGRAMS

T. D. TURNER AND SUSAN L. WIGHTMAN
Pharmacognosy Research Laboratories, University of Wales, Institute of Science and Technology, Cathays Park, Cardiff (Great Britain)
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ortho-Phthalaldehyde (OPT) has been reported by several authors as a chromogenic or fluorogenic reagent. PATTON AND FOREMAN[1] developed a specific colorimetric test for glycine using OPT dissolved in an organic solvent, while CURZON AND GILTHROW[2] and SMITH[3] have employed this reagent for the resolution of several amino acids in paper chromatography.

SHORE, BURKHALTER AND COHN[4] developed a fluorometric method for histamine determination using OPT and SHELLEY AND JUHLIN[5] adapted this method to the resolution of histamine in chromatograms and electropherograms. Using different reaction conditions, MAICKEL AND MILLER[6] used OPT for the fluorometric estimation of certain indoles.

The coloured and fluorescent reactions of OPT were initially considered to be restricted to a few compounds[4], but subsequently it has been shown to react with many substances, among which the following are derived from natural sources: histamine[4]; histidine[2,4]; histidyl histidine[4]; glycine, glutamic acid[2,7]; glutamine, tyrosine[7]; taurine, tryptophan[2,8]; ammonia[4]; glutathione, citrulline[5]; *o*-phenylenediamine[9]; phenylalanine[7,8]; tyramine, dioxyphenylalanine, cysteine, arginine, asparagine, alanine, phenylethylamine, guanidine, monomethylamine, dimethylamine, ethylamine, diethylamine, morphine, codeine, papaverine, heroin, procaine, eserine, solanine, reserpine[8]; yohimbine, apomorphine[8,10]; colchicine, theobromine, arbutine[11]; N-acetyl-5 methoxytryptamine and other substituted indoles[6].

Numerous compounds were reported to give no visible or measurable response to OPT.

CURZON AND GILTHROW[2] noted that the response to OPT was influenced by pH and reaction conditions; and VILLEMIN[11] has reported that in the reaction procedure adopted by SHORE, BURKHALTER AND COHN[4], histamine with OPT produced a yellow colour in alkaline conditions which changed to orange brown when acidified; if carried out in aqueous solution, the reaction produced a rose violet colour. MAICKEL AND MILLER[6] showed that extreme acid conditions were necessary for a fluorogenic reaction between certain indoles and OPT.

RODD[12] gave an account of the activity of OPT and AMOS AND GILLIS[9] characterised the OPT–*o*-phenylenediamine complex, but in general, the nature of the reactions and the structure of the complexes formed by OPT have not been explained.

SHORE, BURKHALTER AND COHN[4] suggested that the histamine OPT reaction was the result of condensation, possibly followed by oxidation, CURZON AND GIL-

THROW[2] and AMOS AND GILLIS[9] have suggested that a Strecker degradation could take place between OPT and amino acids.

The purpose of this paper is to demonstrate that although the nature of the chromogenic and fluorogenic reactions of OPT are not known, this is an effective chromatographic reagent. It reacts with a wide range of substances, particularly if the conditions are varied. It is also shown that OPT is an useful spray reagent for thin layer chromatograms of plant extracts, especially when it is employed as part of a multiple spray system.

EXPERIMENTAL

Thin layer chromatography was used throughout and a comparison was made between the results obtained on three types of thin layer, silica gel, alumina and cellulose.

Aqueous and organic solvents were used. When the solvent system contained ammonia, the chromatoplates were heated at 150° for 10 min before spraying, this removed the ammonia which would otherwise have reacted with OPT to form a grey background[5]. Pyridine and acid fumes did not interfere with the reagent.

Plant extracts were prepared by aqueous extraction of macerated plant tissues, in some cases it was necessary to concentrate extracts *in vacuo* at 40°. Known substances were added to the plant extracts, or were applied in pure solution.

The spray reagent used was that suggested by SHELLEY AND JUHLIN[5], 1 % OPT in *p*-xylene. This remained stable for at least one month in a light proof container, at room temperature. OPT was obtained from British Drug Houses Ltd., and recrystallised from ligroin.

A standard routine was adopted. The chromatoplate was dried in a current of warm air and any coloured spots marked, it was re-examined in a darkroom in U.V. at 254 mμ and 350 mμ and naturally fluorescent areas outlined. The plate remained under the lamp while being sprayed with OPT reagent, and newly fluorescent or changed spots were marked as they became visible. When necessary, an alkaline or acid spray was applied after 2 min and any further changes in fluorescence observed. The plate was then heated in an oven at 110° for 10 min, examined in daylight for colour responses, and re-examined after 12 h for those compounds which showed only a delayed response to OPT.

THE HISTAMINE–OPT REACTION

Sensitivity tests were carried out using three thin layer materials. Aqueous solutions of histamine dihydrochloride and histidine dihydrochloride were prepared by serial dilution with a concentration range between 1 μg and 0.005 μg per 10 μl. A loading of 10 μl of each concentration was applied to silica gel, alumina and cellulose thin layer plates. These were developed with aqueous solvent, and the plates dried and sprayed with OPT reagent.

The sensitivity of OPT reagent to histamine and histidine dihydrochlorides was interpreted as the lowest weight of each substance which gave a visible fluorescence on the developed chromatograms (see Table I).

The response obtained with OPT reagent on histamine showed variations in

fluorescence. Spots less than 0.5 μg loading showed a light blue fluorescence, heavier loadings showed a change from light blue to yellow as the intensity of the fluorescence increased. Histamine spots with a loading greater than 1 μg later developed a yellow spot visible in daylight which increased in intensity as the fluorescence decreased, and remained stable for 24 h.

TABLE I

OPT SENSITIVITY TO HISTAMINE AND HISTIDINE DIHYDROCHLORIDES

Thin layer material	*Minimal loading (μg)*	
	Histamine	*Histidine*
Silica Gel G (Merck)	0.01	0.05
Aluminium Oxide G (Merck)	0.01	0.05
Cellulose (Camag)	0.05	0.1

As stated, VILLEMIN[11] observed that histamine and OPT produced a coloured complex dependent upon pH. These colour changes were investigated and related to their chromatographic response and to the chromogenic resolution of histamine at various pH's.

A 0.2 ml 1 % OPT solution in ethanol was added to 5 ml 1 % histamine dihydrochloride dissolved in buffer solution. Solutions of pH 5 and below were colourless: from pH 6, a pale yellow solution was formed and the colour intensified with increasing alkalinity. On addition of acid to a complex at pH 9, it was found that at pH 8, the yellow colour changed through orange to pink which deepened to magenta below pH 6. On addition of alkali to a colourless acidified solution, the complex became brown-magenta at pH 8.

RODD[12] noted that OPT solution which was made alkaline with ammonia and then acidified, produced an intense red-violet, resembling in description VILLEMIN's results with histamine and OPT. When a comparison was made, OPT with histamine made alkaline then acidified developed a brown-magenta colour, while a similar reaction without histamine showed a grey-brown colour having no resemblance to the histamine–OPT complex.

The coloured histamine–OPT complexes were examined by thin layer chromatography on silica gel with *n*-butanol–acetic acid–water (12:3:5) as the solvent. The yellow alkaline complex showed four components of which two were white fluorescent spots, one intense yellow, not fluorescent, the other orange yellow, not fluorescent. The magenta complex obtained by acidifying the initial alkaline complex showed eight components of which two were fluorescent, white and buff; the remainder nonfluorescent, were yellow, yellow-pink, pink, magenta, yellow-buff and blue-grey. It is probable that the solvent contributed to this multiple response.

The chromogenic response of histamine on thin layer chromatograms with OPT showed a similar dependence on pH. A yellow spot was formed after development on silica gel in a neutral or alkaline solvent; if the OPT reagent was followed by an acid spray, an orange spot was formed. When an acidic solvent, such as *n*-butanol and acetic acid was employed, no coloured response occurred unless an alkaline overspray was applied when a magenta spot appeared after heating.

OPT AS A NON-SPECIFIC LOCATING REAGENT

To improve the range of response to OPT, variations in spray procedure were adopted during the chromatographic examination of many substances. The spray procedure outlined previously was adapted. When an acid and/or alkaline spray was used, it was applied 1–2 min after the initial OPT spray, whilst the plate was being examined in U.V. light.

A spot loading of $2 \cdot 10^{-7}$ moles was applied and the fluorescent and coloured response was noted on chromatograms of silica gel developed with distilled water, using (1) OPT spray reagent alone; (2) OPT followed by 0.02 *N* NaOH; (3) OPT followed by 10% acetic acid, (4) OPT followed by 0.02 *N* NaOH then 10% acetic acid.

After the fluorescent response had been noted, each chromatogram was heated at 110° for 10 min to accelerate the formation of coloured spots.

OPT spray alone

Histamine in pure solution and as a component of plant extracts showed a good fluorescence with the OPT reagent on silica gel and alumina, but was less responsive on cellulose thin layer.

In comparison with the other procedures used, the following substances showed an optimal response to OPT alone on silica gel. With one exception, they formed fluorescent complexes visible at 350 mμ. Heating caused some substances to form coloured spots, and others to disappear completely, and the result of this heat treatment is noted in parentheses: β-alanine (brown); α-amino-*n*-butyric acid (grey); γ-amino-*n*-butyric acid; β-amino-iso-butyric acid (grey); α-amino-octanoic acid (grey-blue); L-arginine monohydrochloride; ethanolamine, at 254 mμ; DL-isoleucine (grey); DL-leucine (green); DL-lysine monohydrochloride (yellow); DL-methionine sulphoxide; DL-methyl histidine (brown); DL-β-phenyl-alanine; DL-serine (yellow); taurine (grey); thiolhistidine (yellow); DL-threonine (yellow); DL-tryptophan (brown); DL-valine (brown).

Sarcosine hydrochloride (grey brown) and L-tyrosine (buff) showed only a coloured response after OPT had been applied and the plate heated.

OPT reagent followed by 0.02 N NaOH

Small amounts of histidine showed an intensified fluorescence after the alkaline overspray. The following substances showed an optimal response to OPT when the alkaline sequel spray was used; the colour assumed after heating at 110° for 10 min is noted in parentheses: 3,5-Di-iodo-L-tyrosine (yellow); L-glutamine (blue-purple); glycine (brown); DL-methionine (yellow); DL-methionine sulphone; pilocarpine nitrate (yellow).

L-Hydroxyproline (brown), DL-norvaline (grey) and DL-ornithine hydrobromide (yellow) showed only a delayed coloured response.

OPT reagent followed by 10% acetic acid

The acid spray caused the OPT fluorescence of many substances to be reduced or quenched, but the following either showed a response to OPT only after spraying with acid, or the initial response to OPT was improved. The colour of the spot, visible

in daylight after heat treatment is noted in parentheses: L-asparagine; DL-citrulline (green); L-cystine (green); 5-hydroxytryptamine (brown).

OPT reagent followed by 0.02 N NaOH then 10 % acetic acid

The OPT reagent was applied, followed by the alkaline spray. The plate was dried in a current of warm air, and then oversprayed with 10 % acetic acid. In most cases this did not improve the initial OPT or OPT–alkali response.

Histamine formed an orange spot, visible in daylight, and histidine, a yellow-green spot. The fluorescence of DL-serine, taurine and DL-threonine was changed to absorbance in U.V. light, and a dark brown spot in daylight. On chromatograms of plant extracts, the acid spray caused much of the natural and OPT fluorescence to be quenched, with no improvement in the formation of coloured spots.

At the beginning of this investigation, the alkaline spray was applied before the OPT reagent[5]. When these were reversed, the following substances gave a positive response when they had previously proved negative: L-arginine monohydrochloride; ethanolamine; glycine; L-hydroxyproline; DL-isoleucine; DL-leucine; L-tyrosine.

The following substances gave a negative response to OPT throughout the tests: DL-α-alanine, DL-aspartic acid, L-glutamic acid, DL-homocysteine, DL-γ-amino-isobutyric acid, L-cysteic acid, acetyl choline, quinine and strychnine.

MULTIPLE SPRAY TECHNIQUE

During the examination of the biological extracts it was apparent that many substances were present which showed no response to OPT, and it was necessary to detect these using other reagents; the comparison of the effects of different reagents on separate chromatograms run under identical conditions was not always satisfactory, and the principle of multiple spray techniques on a single plate, was investigated. The sequence described by SMITH[3] for iminazoles was found to be effective in the examination of plant histamine; this involved:

U.V. ⟶ iodine spray ⟶ U.V. ⟶ ninhydrin then dichloroquinone chloroimide (or reversed) ⟶ anisidine or sulphanilic acid.

It was considered that OPT might be introduced into such a sequence with advantage. But before this could be accomplished it was necessary to evaluate the performance of OPT in double sequence with certain reagents to discover whether the response of either was impaired. The three reagents which had proved most useful in the resolution of plant extracts were used in conjunction with OPT: (1) ninhydrin, (2) iodine reagent, (3) diazotised sulphanilic acid.

OPT with ninhydrin

Ninhydrin reagent was sprayed as 0.5 % w/v solution of indane trione hydrate in *n*-butanol, followed by heating at 105° for 5 min.

When ninhydrin spray was applied first, the response to the OPT spray following this was blocked in substances such as histamine which could show a positive response to both. When OPT was applied as the first spray, a double positive response was obtained with histamine on silica gel thin layers, but not on alumina or cellulose. The ninhydrin response was inhibited if an alkaline spray was applied after OPT, and it

was slightly improved by an acid overspray. The reaction was not reliable, and the colours produced were transient.

The most satisfactory sequence was found to be:

U.V. ⟶ OPT + acetic acid ⟶ heat ⟶ ninhydrin ⟶ heat.

OPT with iodine reagent

Iodine, 1 % in carbon tetrachloride, was used by SMITH[3] as a non-specific reagent for iminazoles, alkaloids, unsaturated acids and phospholipids, and formed yellow, orange or brown spots which were usually transient.

As the first spray in the double sequence, the iodine reagent blocked the response by normally OPT-positive substances which occur in low concentrations in plant extracts; pure histamine and histidine were partially blocked and showed a reduced fluorescence to OPT.

When reversed, OPT was found to give little interference with the iodine response, the OPT fluorescence being replaced by the yellow-brown spots normally seen with iodine reagent, which absorbed at 350 mμ. The following sequence was found to be suitable:

U.V. ⟶ OPT ⟶ heat ⟶ iodine reagent ⟶ U.V.

The carbon tetrachloride solvent was removed by drying in a current of warm air.

OPT with diazotised sulphanilic acid

This has been used extensively as an iminazole reagent, but it has been reported to react also with some phenols and with ammonium salts[3,13].

When OPT was applied as the second spray its response was blocked completely by the strong coupling action of the sulphanilic acid. When the order was reversed, the effect of each reagent was obtained very clearly. The following sequence was used:

U.V. ⟶ OPT ⟶ heat ⟶ diazotised sulphanilic acid.

During these tests on double spray procedures, better responses were obtained when OPT preceded the other sprays.

Multiple sequence

The possibility of including OPT in the multiple spray sequence of SMITH[3] was then assessed using as test substances, histamine, histidine, pilocarpine and plant extracts. The sequence employed was as follows:

U.V. ⟶ OPT ⟶ heat ⟶ iodine reagent ⟶ ninhydrin ⟶ heat ⟶ diazotised sulphanilic acid.

Ninhydrin proved to be unreliable and a modified sequence was adopted:

U.V. ⟶ OPT ⟶ heat ⟶ iodine reagent ⟶ diazotised sulphanilic acid.

Histamine, histidine and pilocarpine showed a normal positive response to each reagent in succession, and the natural histamine and histidine content of plant extracts reacted similarly. Other constituents of these extracts, normally responsive to each of these reagents, could also be detected.

DISCUSSION

This investigation originated in the need to examine the composition of plant extracts which were to be submitted for fluorometric analysis; thin layer chromatography was the most suitable technique and it was convenient to use the same fluorogenic agent, OPT, in both procedures. Thus several OPT positive substances were discovered in plant extracts where only histamine and histidine had been anticipated. This led to a study of the more general use of OPT for the resolution of chromatograms.

Several authors have extended the known range of OPT active substances and have expressed the opinion that many other compounds may also react to this reagent. In this investigation the range is further extended to include the following compounds: α-amino-*n*-butyric acid, γ-amino-*n*-butyric acid, β-amino-isobutyric acid, α-amino-octanoic acid, cystine, 3,5 di-iodo-L-tyrosine, ethanolamine, hydroxyproline, isoleucine, leucine, lysine, methionine, methionine sulphone, methionine sulphoxide, norvaline, ornithine, sarcosine, serine, threonine, valine, methyl histidine, thiolhistidine and pilocarpine.

The diversity of response to this reagent should be emphasised. The visible OPT complex has been observed in solution, on gel preparations, on paper and thin layer chromatographic materials; it is not permanent but its effect can be fluorogenic and chromogenic.

Some reactive substances form only a fluorescent complex *e.g.* ethanolamine, β-phenylalanine and arginine; others produce a coloured response, *e.g.* sarcosine, tyrosine and hydroxyproline. Most substances show a fluorescent response which is then replaced by a less transient coloured complex; in some cases, substances with similar fluorescence can be distinguished by their chromogenesis, for example, leucine and isoleucine. These distinctions can be emphasised by the use of an acid or alkaline overspray.

We suggest that OPT is a useful chromatographic reagent because it is non-specific, effects a dual response, and is sensitive to variation in the conditions of reaction. The extent of these variations has not been explored, and the optimal conditions for the resolution of thin layer chromatograms with OPT cannot be detailed until the nature of its chromogenic and fluorogenic reactions has been elucidated.

The double and multiple spray sequences which are described are most effectively applied to the examination of iminazoles. It is unfortunate that ninhydrin failed to show a consistent response in conjunction with OPT, as this could have extended the general function of the multiple spray sequence and might have increased the range of application of OPT as a chromatographic reagent.

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SUMMARY

The use of *o*-phthalaldehyde (OPT) as a non-specific reagent to resolve thin layer chromatograms is investigated and the range of known reactive substances

increased by varying the conditions of the reaction. The inclusion of OPT reagent in multiple sequence is examined and a sequence of reagents for iminazoles is suggested.

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