STUDIES ON THE NATURE OF SOME CHROMATOGRAPHIC MULTISPOTS FROM ADRENALINE

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Pure adrenaline[1] and other tissue amines[2] gave two spots when chromatographed on paper in the presence of trichloroacetic and certain other acids. The phenomenon was attributed to the formation of unstable complexes, between the amines and the acids, of the type previously described for weakly acidic phenols[3]. Double spot formation by sympathomimetic amines was further investigated by BECKETT, BEAVAN AND ROBINSON[4,5] who showed that one of the amine spots corresponded to the free base while the other was associated with the acid. They demonstrated that the major factors involved in the phenomenon were the relative proportions of, and R_F values of, the amines and the added acids and also the relative local concentrations of, and dissociation constants of, the added acid and that present in the solvent system.

More recently[6,7] the formation of multiple spots by β-phenylethylamine derivatives when chromatographed from 10 N hydrochloric acid has been reported. This phenomenon appeared to be unrelated to that previously described in that the same acid (hydrochloric) was used in the developing solvent as was applied with the amine. Furthermore the presence or absence of the phenomenon and the absolute number of extra spots obtained was shown to be related to the chemical structures of the individual amines. Hydrochloric acid is often used during the preparation of extracts of biological tissues and fluids prior to chromatography, and pure adrenaline exposed to these extraction techniques has been shown to form multispots[8]. Since some of the substances responsible for the extra spots could therefore appear on chromatograms of tissue extracts containing sympathomimetic catecholamines, we were interested in elucidating their chemical structures. Adrenaline was the parent amine chosen for the investigation because it has given the optimal number of discrete multispots[7].

EXPERIMENTAL

Paper chromatography

The apparatus, materials and techniques used for single and two dimensional paper chromatography have been previously described[6,9]. Adrenaline, diadrenaline ether, adnamine and adrenochrome were chromatographed on Whatman No. 1

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paper (washed with 0.01 *N* HCl) from a solution in distilled water or hydrochloric acid (10 *N*). The ascending developing solvent was phenol containing 15 % v/v 0.1 *N* HCl, and potassium ferricyanide (0.5 g) in sodium hydroxide solution (100 ml, 0.5 *N*) was used to locate the amines.

Thin layer chromatography

All plates used were 5 × 20 cm. They were air dried for 24 h before use.

Cellulose. 15 g Whatman Standard Grade Cellulose and 75 ml of a 1 in 3 dilution of mucilage of tragacanth. Thickness 1 mm.

Methyl cellulose. 10 g Gelacol (M20) and 25 ml 95 % ethanol. Thickness 0.25 mm.

Silica gel. 50 g Kieselgel H and 100 ml distilled water. Thickness 0.25 mm.

Alumina. 25 g Alumina Type H 100/200 mesh and 35 ml of a 1 in 3 dilution of mucilage of tragacanth. Thickness 0.25 mm.

The solvent system and spray reagent used were the same as for the paper chromatography.

Compounds chromatographed. (—)-Adrenaline base, adrenochrome base (L. Light & Co. Ltd.) and (—)-adrenaline acid tartrate (Burroughs Wellcome & Co.) were obtained commercially.

Diadrenaline ether hydrochloride was synthesised according to the method of OPPINGER AND VETTER[10], and the infra-red spectrum of this compound is shown in Fig. 1. The reaction mechanism must presumably involve the replacement of the side chain hydroxyl group of one molecule of adrenaline by chlorine which then reacts with another molecule of adrenaline.

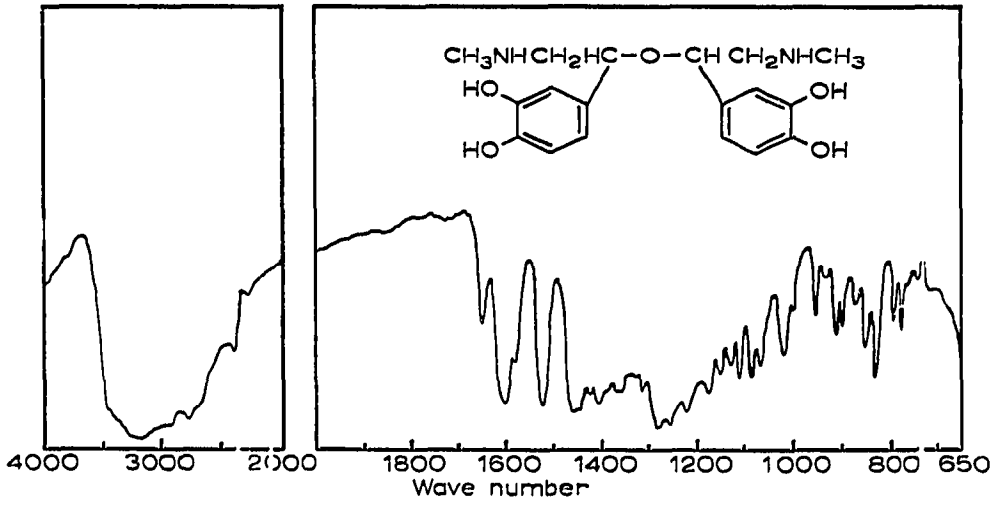

Fig. 1. Infrared spectrum of diadrenaline ether hydrochloride (KCl disc).

Adnamine hydrochloride was synthesised using the method described below, since in our hands the experimental conditions given by KAWAZU[11] for the synthesis of this compound resulted in the formation of a substance identical to the diadrenaline ether of OPPINGER AND VETTER[10]. (—)-Adrenaline base (5 g) was refluxed at 140° in HCl (12.5 ml, 20 % w/v) until crystals were formed (approx. 12 h). The reaction mixture was filtered hot through a pre-heated sintered glass filter and the crystals washed several times with hot 20 % w/v HCl. After final washings with ice-cold

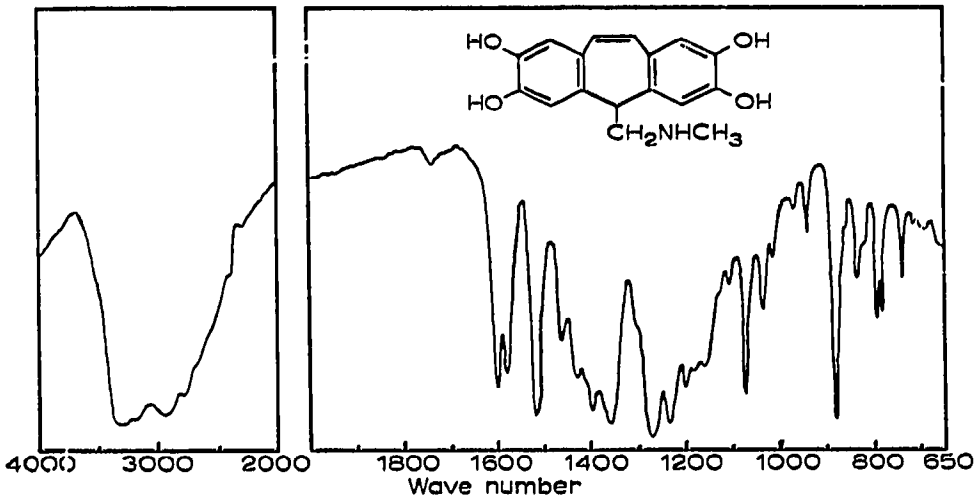

Fig. 2. Infrared spectrum of adnamine hydrochloride (KCl disc).

distilled water followed by ice-cold ethanol approximately 1 g (dry weight) of crystals was obtained. The infra-red spectrum of this compound (Fig. 2) differed from that of diadrenaline ether (Fig. 1) and was identical with that of adnamine hydrochloride[11].

RESULTS AND DISCUSSION

As demonstrated previously[7] single dimensional development on paper of solutions of adrenaline (100 μg) in 10 N hydrochloric acid (0.01 ml) resulted in the formation of multispots, the chromatographic characteristics of which depended upon the age of the solution (Fig. 3). In the present study two dimensional chromatograms of similar solutions showed the same number of spots and in each case the colours and locations of these spots following development in the second direction were characteristic of the colours and locations of the spots obtained in the first direc-

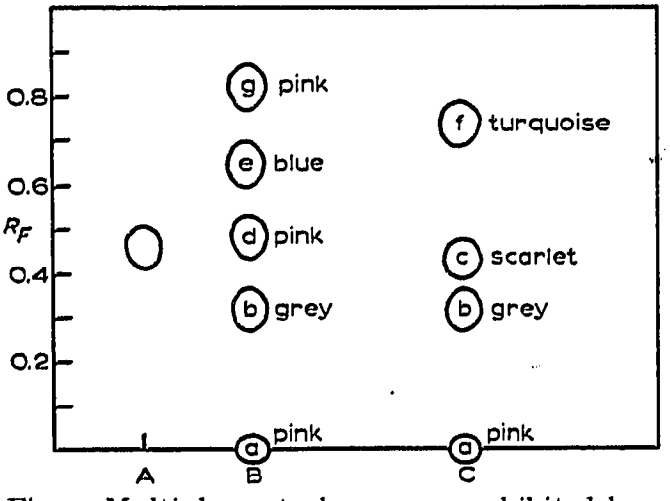

Fig. 3. Multiple spot phenomena exhibited by adrenaline acid tartrate (200 μg) when chromatographed from hydrochloric acid (10 N) immediately after (B), and 7 days after (C), preparing a 10 mg/ml solution. At A, adrenaline acid tartrate chromatographed from solution in distilled water. Developing solvent, phenol containing 15% v/v 0.1 N HCl. The spots are labelled a–g to correspond with the text.

tion. Similarly, when distilled water eluates prepared from strips cut at the multispot R_F values were concentrated under reduced pressure and rechromatographed, with one exception, discrete spots were obtained from each eluate having colour reactions and R_F values characteristic of the appropriate multispot from which they were prepared. This was taken to indicate that the multiple spots resulted from the formation of stable compounds of definite chemical structure. The exception was spot a (base-line retention) which was not evident subsequent to elution and re-chromatography. When each strip of eluted paper was routinely tested for residual amine by spraying with alkaline ferricyanide, however, strips cut at the base-line showed pink areas corresponding exactly with the area to which the solution had been originally applied. Colour reactions were absent from eluted strips cut at other levels on the paper. This phenomenon was given rationale when solutions of adrenaline in 10 N HCl were chromatographed on thin layer plates. Retention at the application point was only evident on those plates containing cellulose although the other multiple spots were best demonstrated on the silica gel plates.

In the presence of 10 N HCl the β-hydroxyl group on some of the adrenaline molecules is undoubtedly chlorinated and this could result in the formation of ether linkages with hydroxyl groups present in cellulose in a reaction analogous to the formation of diadrenaline ether (see Experimental). Adrenaline bound to cellulose in this way would be unable to migrate during chromatographic development and would result in retention at the point of application (spot a).

From the literature we had ascertained that diadrenaline ether and adnamine were known products of reaction between adrenaline and hydrochloric acid and chromatography of these compounds from solution in distilled water established that they were responsible for spots b and c, respectively. When the same two compounds were chromatographed from a fresh solution in 10 N HCl their R_F values were unchanged and no additional spots were evident. Since spot d was obviously adrenaline itself at its normal R_F value, we were now left with spots e, f and g to investigate.

Because the results of some previously reported experiments had indicated the possibility of oxidation of adrenaline in the presence of concentrated hydrochloric acid[8], the chromatographic behaviour of adrenochrome in aqueous and acid solution was investigated. Chromatography from distilled water yielded a single spot which corresponded to spot g, whereas chromatography from solution in 10 N HCl produced two spots having R_F values and colour reactions characteristic of spots e and g. In both cases a grey streak from the starting point to the solvent front was evident on the unsprayed developed chromatograms. The obvious inference from these results is that spot g is adrenochrome and that spot e is some derivative of adrenochrome formed in the presence of hydrochloric acid. This interpretation assumes that oxidation of adrenaline has occurred in the presence of 10 N HCl; the influence of antioxidants on the multiple spot phenomena in 10 N HCl was therefore investigated.

In confirmation of previous results[8], the presence of pyrogallol (10 mg/ml 10 N HCl) prevented the formation of all of the artifact spots normally seen when adrenaline was chromatographed from 10 N HCl. Our present investigation of this phenomenon, however, has shown that it results not from the prevention of oxidation, but from the formation of a complex between pyrogallol and adrenaline in the presence of 10 N HCl, thus removing the source of the artifact spots. By contrast the presence of sodium metabisulphite (1 mg/ml 10 N HCl) failed to prevent the formation of any of the extra

spots. These results were also invalid, however, since SO_2 gas was evolved when the sodium metabisulphite was added to the 10 *N* HCl and the sodium chloride formed as a consequence is not expected to have anti-oxidant properties. Finally we prepared ferrous chloride[12] and found that it remained at the starting point under our chromatographic conditions and did not complex with adrenaline. At a concentration of 100 mg/ml 10 *N* HCl, this freshly prepared ferrous chloride prevented the formation of spots e, f and g only. We concluded, therefore, that oxidation was involved in the formation of these three spots.

Under our highly acid conditions, however, protonation of the nitrogen will prevent cyclization and any oxidation of adrenaline will stop at adrenaline quinone[13].

As the developing solvent passes over the application area, however, dilution of the acid will occur; if the pH rises above 2 then cyclisation will occur immediately with the formation of leucoadrenochrome[14]. This may well behave chromatographically like adrenochrome, to which it will be certainly converted when the paper is sprayed with the alkaline ferricyanide. We therefore suggest that spot g is leucoadrenochrome, and that spot e, being presumably formed from it in the presence of concentrated hydrochloric acid, could be its dehydration product, 5,6-dihydroxy-N-methylindole.

Information concerning the structure of spot f was obtained after we remembered that this spot only occurred on chromatograms of acid solutions of adrenaline at least 24 h old[7]. When we accordingly investigated the chromatographic behaviour of 48 h solutions of adnamine and diadrenaline ether in 10 *N* HCl, although adnamine still showed only one spot (c), diadrenaline ether now produced two (b and f). Since the prior addition of ferrous chloride to the 10 *N* HCl prevents the formation of spot f from diadrenaline ether, we again suggest that cyclisation has taken place and that spot f is dileucoadrenochrome ether which will be converted to diadrenochrome ether by the alkaline ferricyanide spray reagent. The possibility of oxidation of sympathomimetic amines in acid solution warrants further investigation, particularly since spots e, f and g can be formed from monophenolic amines, substances reported not to form adrenochrome-like oxidation products.

The final significance of our results will depend upon the results of our present pharmacological investigations of the compounds responsible for the artifact spots. Certainly when hydrochloric acid is used during the production of concentrated extracts of biological material for chromatography, the number of spots obtained need not necessarily indicate the number of amines present in the starting material. Furthermore the pharmacological activity of the artifact substances, in addition to interfering with the bioassay of naturally occurring amines present in chromatographic eluates, might result in erroneous conclusions concerning the existence of biologically active metabolites. In this context it is noteworthy that spots e and f have R_F values similar to that of isoprenaline in the same solvent system.

SUMMARY

An attempt has been made to identify the substances demonstrable as seven multispots when adrenaline is chromatographed on paper from hydrochloric acid solution. The evidence for considering these substances to be (a) adrenaline bound to the paper, (b) diadrenaline ether, (c) adnamine (5-methylaminomethyl-2,3,7,8-

tetrahydroxydibenzo-[*a*,*e*]-cycloheptatriene), (d) adrenaline, (e) 5,6-dihydroxy-N-methylindole, (f) dileucoadrenochrome ether and (g) leucoadrenochrome, respectively, is discussed.

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