Journal of Chromatography, 79 (1973) 243–249

CHROM. 6584

DOUBLE-SPOT FORMATION IN THE THIN-LAYER CHROMATOGRAPHY OF PURE FENFLURAMINE HYDROCHLORIDE AND SOME OTHER AMINES IN THE PRESENCE OF ORGANIC SOLVENTS

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(First received September 29th, 1972; revised manuscript received January 8th, 1973)

SUMMARY

From a number of solutions of fenfluramine hydrochloride in (a) ethanol or (b) chloroform, chromatographed on thin layers of silica gel or alumina, using the solvent systems chloroform–methanol (1:1), methanol–acetone (1:1) and chloroform–ethanol (4:1), two spots were produced, whereas solutions in water gave only one spot. This phenomenon did not occur when the sulfate salts of the amines were used. Similar results were obtained with amphetamine and ethylamphetamine hydrochlorides. The material extracted from the faster moving spots was identified as fenfluramine hydrochloride and that from the lower spots as fenfluramine. Partial and complete hydrolysis, depending on the ionic strength of the acid, were factors leading to the formation of double spots (ethanolic solutions) and single spots (aqueous solutions) of the amine hydrochlorides.

INTRODUCTION

Multiple spot formation by sympathomimetic amines and some other compounds in paper chromatography[1–7] and thin-layer chromatography (TLC) on cellulose[8–10] has been reported on several occasions. Multiple spots were obtained when compounds were chromatographed from solutions in hydrochloric and other acids stronger than those of the solvent system, using a neutral or acidic solvent system.

In the present work we have examined our observations that pure fenfluramine hydrochloride [1-(3-trifluoromethylphenyl)-2-ethylaminopropane] gives two spots when it is chromatographed on thin layers of silica gel and alumina using various organic solvents.

Different amino-based drugs may be used in pharmaceutical preparations either as bases or as salts. These drugs, when chromatographed, may give double spots which could lead to erroneous conclusions with respect to the purity of such drugs and their suitability in pharmaceutical and medicinal practice. We have investigated the quantitative composition of the two spots and attempted to elucidate the mechanism of double-spot formation.

EXPERIMENTAL

Materials

Fenfluramine hydrochloride, fenfluramine sulfate, fenfluramine base, ethylamphetamine hydrochloride, amphetamine hydrochloride and amphetamine sulfate were used. All chemicals used were of pharmacopoeial or analytical grade or of equivalent purity.

Developing systems. (a) Chloroform–methanol (1:1); (b) methanol–acetone (1:1) (c) chloroform–ethanol (4:1).

Spray reagents. The spots were detected using (a) Dragendorff's reagent, (b) diazotized *p*-nitroaniline–sodium hydroxide reagent, or when necessary with (c) silver nitrate solution[11].

Thin-layer plates. All plates were prepared from Silica Gel G (Merck) and Aluminum Oxide G (Merck) as described by STAHL[12]. The layer thickness was 0.75 mm for preparative chromatographic experiments and 0.25 mm for all other purposes.

Scintillation solvent. A 5-g amount of 2,5-diphenyloxazole (PPO), 0.3 g of dimethyl-1,4-bis[2-(5-phenyloxazolyl)benzene] (dimethyl-POPOP) and 35 g of Cab-O-Sil were dissolved in 1 l of toluene.

General procedure

A 10-μl volume of the compounds was added to silica gel or alumina plates from freshly prepared solutions (10 mg/ml) in absolute ethanol, distilled water or various organic solvents, and the chromatograms were developed at room temperature (about 22°).

Methods of preparative TLC for identification of the double spots

Fenfluramine hydrochloride (100 mg) in absolute ethanol was spotted on to two glass plates with silica gel layers of 0.75 mm thickness. After development in solvent system (a), the spots were located by spraying the detector lanes with Dragendorff's reagent, scraped off, eluted with absolute ethanol and the solvent was removed. The substances so obtained were chromatographed on thin layers of silica gel using solvent systems (a), (b) and (c) and Dragendorff's reagent or diazotized *p*-nitroaniline.

Spectra

UV and IR spectra were measured of materials extracted from the spots for identification purposes.

Spectrophotometric measurements were carried out with a Hitachi Perkin-Elmer spectrophotometer, Model 139, with 1-cm cells and a Perkin-Elmer 257 grating IR spectrophotometer using potassium bromide pellets or chloroform solutions.

Methods for quantitation of the double spots

The quantitation of the spots was carried out by indirect spectrophotometric measurements in the UV region after the substances had been scraped off the silica gel thin layers and eluted.

A standard solution of fenfluramine hydrochloride in absolute ethanol was prepared (50 mg/ml), and volumes of 10, 30, 50, 70 and 100 μl were applied to thin layers

of silica gel and the chromatograms were obtained in the usual manner. After drying the plates, the spots were located by spraying the detector lanes with Dragendorff's reagent, the corresponding squares of adsorbent were removed by careful scraping and transferred to 20-ml conical flasks. The substances were eluted from the adsorbent by adding 5 ml of absolute ethanol and shaking well for about 8–10 min. The suspensions were centrifuged and the upper layers transferred into 10-ml calibrated flasks; absolute ethanol was added to volume. No fenfluramine was detected on the silica gel residue.

A blank sample of the adsorbent was treated in a similar manner.

The UV absorption of each solution was measured at 262 nm and the values were compared with the standard curve, which was constructed by using 25–750 μg/ml concentrations of the stock solution (after reducing the absorption values due to the blank, if necessary).

Liquid scintillation counting and chromatogram scanning

The distribution and quantitative evaluation of the chloride ions in the spots of the thin-layer chromatograms of fenfluramine hydrochloride were carried out by using radioactive chlorine-36. Fenfluramine base (25 mg) was dissolved in 1.0 ml of absolute ethanol and 0.01 ml of hydrochloric acid labelled with ^{36}Cl was added. The precipitated salt was crystallized from hot ether and the purity compared with the standard fenfluramine hydrochloride.

A standard solution of radioactive fenfluramine hydrochloride in absolute ethanol was prepared (25 mg/ml) and volumes of 1, 2, 3, 5, 10, 20 and 30 μl were applied to thin layers of silica gel and solvent system (a) was used. The marked areas of the two spots were removed and introduced into 20-ml scintillation-counting vials containing 10 ml of the scintillation solvent. The radioactivity of each sample was determined in a Packard Model 3320 Tri-Carb spectrometer. Controls for background and known amounts of labelled fenfluramine hydrochloride were run concurrently to serve as a check on the technique and the performance of the counter.

Known concentrations of 25–750 μg of fenfluramine [^{36}Cl]hydrochloride were carried through the procedure without developing them on the thin layers, and a linear relationship was obtained with the curve passing through the origin when net counts per minute (c.p.m.) were plotted against the concentration. From the graph, c.p.m. values were converted into concentrations for use in the calculation of the material present in the two spots developed.

The direct method of scanning the thin-layer chromatograms of radioactive fenfluramine hydrochloride was used with a Packard radiochromatogram scanner, Model 7201, using 5, 10, 20 and 30 μl of the standard solutions on the thin layers of silica gel. After the chromatograms had been developed, the plates were placed in the instrument for scanning. Known amounts of fenfluramine [^{36}Cl]hydrochloride standard solution were spotted on to separate plates of silica gel thin layers and their radioactivity was measured without developing them. The results were used for calculating the radioactivity of the material present in the two spots.

An aqueous solution of fenfluramine [^{36}Cl]hydrochloride (25 mg/ml) was also prepared, and volumes of 5, 10, 20 and 30 μl were treated as described above for the ethanolic solutions using solvent system (a).

RESULTS

Using silica gel thin layers on glass plates, solutions of fenfluramine hydrochloride in absolute ethanol or chloroform produced two completely separated spots with R_F values of 0.30 and 0.72 using solvent systems (a), (b) or (c). One of the spots was in the same position as fenfluramine base dissolved in absolute ethanol or chloroform (R_F value 0.30). However, when an aqueous solution of fenfluramine hydrochloride was used, it produced one spot (R_F value 0.30). The more intense of these spots were obtained at the higher R_F value. Double-spot phenomena with varying distances between the spots similar to the above were obtained when solutions of fenfluramine hydrochloride in acetone, ethyl acetate and benzene were used (Fig. 1); these results are summarized in Table I. Almost identical results were obtained with fenfluramine hydrochloride solutions in absolute ethanol, chloroform and water when alumina thin layers were used, but the R_F values were different.

TABLE I

R_F VALUES OF FENFLURAMINE HYDROCHLORIDE IN DIFFERENT SOLVENTS ON SILICA GEL THIN LAYERS WHEN CHROMATOGRAPHED IN CHLOROFORM–METHANOL SOLVENT

Solvent	*R_F value*	
Absolute ethanol	0.70	0.31
Chloroform	0.68	0.30
Acetone	0.60	0.30
Ethyl acetate	0.48	0.28
Benzene	0.45	0.23
Water	—	0.26

When fenfluramine sulfate was dissolved in absolute ethanol or chloroform and chromatographed on silica gel plates using solvent system (a), it produced only one spot with an R_F value identical with that of the higher spot of fenfluramine hydrochloride (Fig. 2). Amphetamine and ethylamphetamine hydrochlorides gave two spots when their solutions in ethanol or chloroform were chromatographed on silica

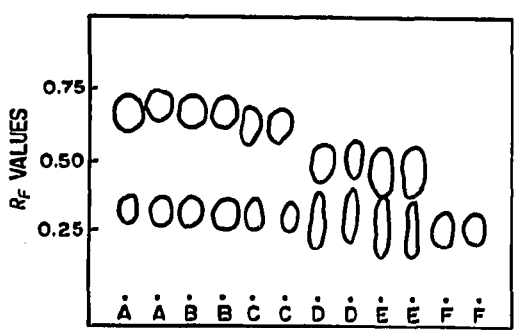

Fig. 1. Thin-layer chromatogram of freshly prepared solutions of fenfluramine hydrochloride in ethanol (A), chloroform (B), acetone (C), ethyl acetate (D), benzene (E) and water (F) on silica gel when solvent system (a) is used.

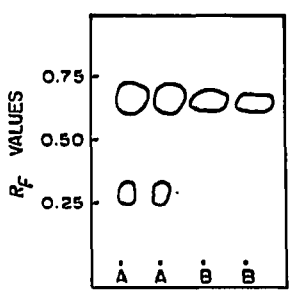

Fig. 2. Thin-layer chromatogram of freshly prepared solutions of fenfluramine hydrochloride (A) and fenfluramine sulfate (B) in ethanol on silica gel when solvent system (a) is used.

gel and alumina thin layers, but in aqueous solutions only one spot was produced, with the same R_F value as that of the free base. The sulfate salts of amphetamine and ethylamphetamine gave only one spot (Fig. 3).

To locate the hydrochloric acid spot of the salt solutions, the developed chromatograms of fenfluramine hydrochloride in ethanol were sprayed with silver nitrate reagent. From the location of the white precipitate, it was evident that the faster moving spots (R_F about 0.70) contained hydrochloric acid. Hydrochloric acid alone in the same systems gave spots with R_F values of 0.68–0.70.

Using preparative TLC, a white crystalline powder from the upper spot and only small amounts of a light yellowish green oil from the lower spot were obtained.

The extracted materials from the upper spots gave two spots when their freshly prepared solutions in absolute ethanol were added to silica gel plates in systems (a), (b) and (c). Their R_F values were identical with those of the upper and lower spots of fenfluramine hydrochloride. The material extracted from the lower spots gave only a single spot with an R_F value identical with that of fenfluramine base (Fig. 4).

The UV and IR spectra of the material extracted from the lower and upper spots were identical with those of fenfluramine base and fenfluramine hydrochloride, respectively.

The melting-point of the material extracted from the upper spot was identical with that of an authentic sample of fenfluramine hydrochloride (168.5–169°).

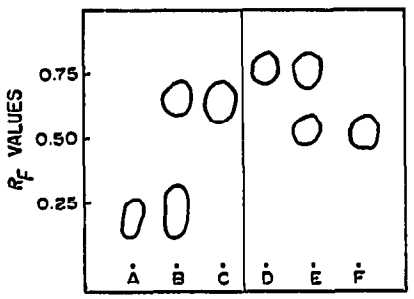

Fig. 3. Thin-layer chromatograms of freshly prepared solutions of amphetamine sulfate (A), amphetamine hydrochloride (B), amphetamine base (C), ethylamphetamine sulfate (D), ethylamphetamine hydrochloride (E) and ethylamphetamine base (F) in ethanol on silica gel when solvent system (a) is used.

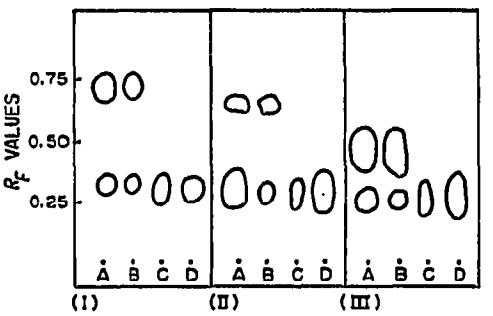

Fig. 4. Thin-layer chromatograms of freshly prepared solutions of fenfluramine hydrochloride in ethanol (A); upper spot obtained from preparative chromatography of fenfluramine hydrochloride in ethanol (B); lower spot of fenfluramine hydrochloride obtained in the same manner, in ethanol (C); fenfluramine base in ethanol (D) on silica gel when solvent systems (a) I, (b) II and (c) III were used.

Recoveries of fenfluramine from the spots

The recovery of fenfluramine from the spots was determined from the standard curve of fenfluramine hydrochloride and tabulated, using the mean values from three consecutive measurements (Table II). The recoveries of fenfluramine (the sum of the contents in the two spots) were virtually quantitative.

Determination of radioactivity in the spots

The recovery of ^{36}Cl from the faster running spots ranged between 63.1 and

TABLE II

RECOVERY OF MATERIALS EXTRACTED FROM THE TWO SPOTS AND CALCULATED AS FENFLURAMINE HYDROCHLORIDE USING AN ULTRAVIOLET SPECTROPHOTOMETER

Amount spotted on plates (μg)	*Theoretical absorptivity*	*Mean absorptivity after elution*		*Recovery of material as fenfluramine hydrochloride (μg)*	
		Upper spot	*Lower spot*	*Upper spot*	*Lower spot*
20	0.04	0.03	0.01	15	5
60	0.12	0.11	0.015	55	7.5
100	0.20	0.19	0.015	92.5	7.5
140	0.285	0.27	0.020	132.5	10.5
200	0.400	0.38	0.025	187.5	12.5

87.7 %. The substance collected from the lower spot was not radioactive, *i.e.*, hydrochloric acid was not present in the lower spot.

The determination of radioactivity directly on the thin layers on the glass plates gave similar results. However, the single spots from aqueous solutions of fenfluramine [^{36}Cl]hydrochloride were not radioactive, but there was strong radioactivity and a negative Dragendorff reaction in the space above these spots at the level of the higher running spots of ethanolic solutions of the salt; the amount of radioactivity was 87–88 % of the total radioactivity of the spotted samples. This radioactivity must arise from hydrochloric acid derived from complete hydrolysis when fenfluramine hydrochloride is added to the thin layer in aqueous solution.

DISCUSSION

The two spots obtained on TLC of pure fenfluramine hydrochloride on silica gel or alumina were identified by IR and UV spectroscopy as the amine hydrochloride in the faster moving spot and the amine in the lower spot; much more of the amine was present in the upper than in the lower spot. The total amine added to the plates was virtually completely recovered from the spots.

It was concluded that the two spots that were present when organic solvents, but not when water, were used arise from the partial solvolysis of the amine hydrochloride. This conclusion is supported by the fact that when the strength of the acid salt was increased and a less volatile acid was used, only one spot was formed. Using an aqueous solution, there was only one spot of amine hydrochloride and this was located at the position of the free amine, suggesting complete hydrolysis in water.

Because the chloride was not recovered completely, it is assumed that in solvents in which partial hydrolysis had occurred, some of the free hydrochloric acid volatilized during the running of the system.

The use of radioactive [^{36}Cl]hydrochlorides of the amines showed the absence of chloride ions in the lower spots.

Thus, the extent of hydrolysis increased from partial hydrolysis in the ethanolic solutions to complete hydrolysis when the salts were in aqueous solutions.

The formation of double spots in non-polar organic solvents, such as chloroform,

may perhaps be attributed to protolysis that occurs with the anion of the salt and the organic solvent.

As has been stressed earlier[13], the siloxane and silanol groups in silica gel and the negative charge on the oxygen atoms in alumina may perhaps also interfere here with the interaction of the protonated or non-protonated amines in the aqueous or ethanolic solutions of amine hydrochlorides and make their contribution to the binding of amines through anion–cation association and the formation of the two spots.

It is therefore concluded that the formation of two amine spots when an amine hydrochloride is subjected to TLC on silica gel and alumina results from the following: (a) partial hydrolysis of the amine salts, depending on the ionic strength of the acid; (b) volatilization of the liberated hydrochloric acid; and (c) the presence of charged groups in silica gel and alumina thin layers.

This investigation shows that the presence of more than one spot in silica gel and alumina thin-layer chromatograms of amine hydrochlorides in ethanol and some other organic solvents is not indicative of lack of purity of the drugs. It also reveals that the single spot of fenfluramine and other amine hydrochlorides in water when chromatographed under the above conditions represents the base developed after complete hydrolysis.

ACKNOWLEDGEMENTS

The author gratefully acknowledges financial support for this work from the Department of Pharmacy, School of Pharmacy, University of North Carolina. Thanks are due to Professor A. M. MATTOCKS, Dr. E. J. TRIGGS, and JERRY CARLSON, School of Pharmacy, University of North Carolina, for useful suggestions and discussions. The author thanks Professor A. H. BECKETT, Chelsea College, University of London, for supplying samples of the amines and his detailed criticism of the manuscript.

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