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ISOTOPIC FRACTIONATION IN THIN-LAYER CHROMATOGRAPHY

HENRY D'A. HECK*
Mass Spectrometry Research Center, Stanford Research Institute, Menlo Park, Calif. 94025 (U.S.A.)
ROBERT L. SIMON
Organic Chemistry Group, Stanford Research Institute, Menlo Park, Calif. 94025 (U.S.A.)
and
MICHAEL ANBAR
Mass Spectrometry Research Center, Stanford Research Institute, Menlo Park, Calif. 94025 (U.S.A.)
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SUMMARY

The thin-layer chromatography of imipramine on silica gel plates was studied in fifteen solvent systems. The mobility of imipramine labeled with deuterium in the methyl groups of the dimethylaminopropyl side chain differs markedly from that of unlabeled imipramine. Partial or complete separations between unlabeled and deuterated imipramine were observed in all basic and neutral solvent systems investigated, but not in weakly acidic solvents. Isotopic fractionations of imipramine were also found on alumina thin-layer plates, but were not detected in cellulose chromatography. In all thin-layer isotopic separations, the unlabeled compound migrates more rapidly than the deuterated molecule. These results can be explained by a stronger basicity of deuterated imipramine relative to its unlabeled counterpart.

INTRODUCTION

Numerous examples of isotopic fractionation in liquid and gas chromatographies have been reported[1–14]. In contrast, few accounts of this phenomenon exist in the literature of thin-layer chromatography (TLC). Viswanathan[15] reported an apparent $^{14}C/^{12}C$ fractionation effect in the chromatography of sodium formate on silica gel plates whose magnitude is so large as to bring into question the purity of the ^{14}C-labeled material used. In the only other case known to us, Gold and Crigler[16] demonstrated that certain [1,2-^{3}H]steroids migrate slightly more slowly than the corresponding unlabeled or ^{14}C-labeled compounds on silica gel plates.

While preparing a calibration curve for the determination of imipramine (5-[3-(dimethylamino)-propyl]-10,11-dihydro-5H-dibenz[*b*,*f*]azepine) in human plasma by stable isotope dilution analysis, it was discovered that unlabeled imipramine

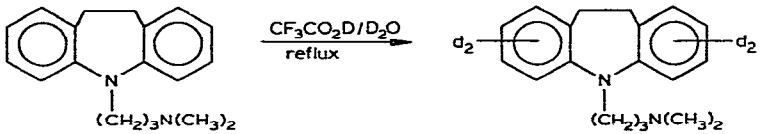
* To whom correspondence should be addressed.

and imipramine deuterated in the methyl groups of the dimethylaminopropyl side chain were separated from each other completely by TLC on silica gel. The unexpected separation of the two isotopic variants could not be ascribed to nonisotopic differences, as the structures of the variants were otherwise identical, according to several chromatographic and spectroscopic criteria. The details of these experiments and the chromatographic properties of the labeled and unlabeled compounds are the subjects of this report.

EXPERIMENTAL

Chemicals

Unlabeled imipramine hydrochloride was a gift of Ciba/Geigy (Basle, Switzerland). Imipramine-d_4 was synthesized from the unlabeled compound by an acid-catalyzed exchange in the presence of D_2O. Unlabeled imipramine hydrochloride was heated under reflux in a solution of deuterated trifluoroacetic acid in D_2O for 48 h (reaction 1). The reaction was quenched with sodium bicarbonate and the



Imipramine (m/e 280) Imipramine-d_4 (1)

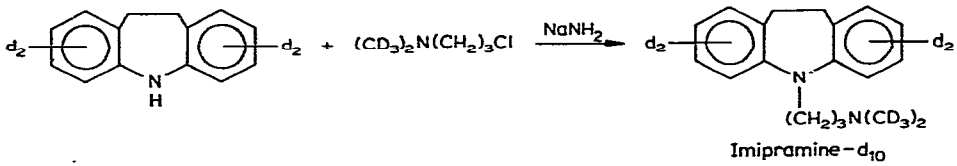
product was extracted into diethyl ether. The hydrochloride salt was formed by treatment with HCl gas, and the product was recrystallized from acetone.

A nonfragmented mass spectrum of imipramine-d_4 was obtained using a mass spectrometer constructed in this laboratory equipped with a multipoint field ionization source[17]. The spectrum is presented in Fig. 1 and includes the observed d_0/d_4 ratio not corrected for the background. The low value of this ratio indicates that the exchange proceeded virtually to completion.

Multilabeled imipramine containing deuterium in the methyl groups of the dimethylaminopropyl side chain, as well as in the dibenzazepine moiety, was synthesized by the routes shown in reactions 2–4.

$$(CD_3)_2NH + CH_2{=}CHCH_2OH \xrightarrow[\Delta]{NaOH} (CD_3)_2N(CH_2)_3OH \quad (2)$$

$$(CD_3)_2N(CH_2)_3OH + SOCl_2 \xrightarrow[\Delta]{} (CD_3)_2N(CH_2)_3Cl \cdot HCl \quad (3)$$



Imipramine-d_{10} (4)

To prepare N-dimethyl-3-chloropropylamine hydrochloride (reactions 2 and 3), allyl alcohol was heated to 120° in a sealed glass autoclave that contained dimethylamine-d_6 and sodium hydroxide. After 20 h, the reaction mixture was quenched

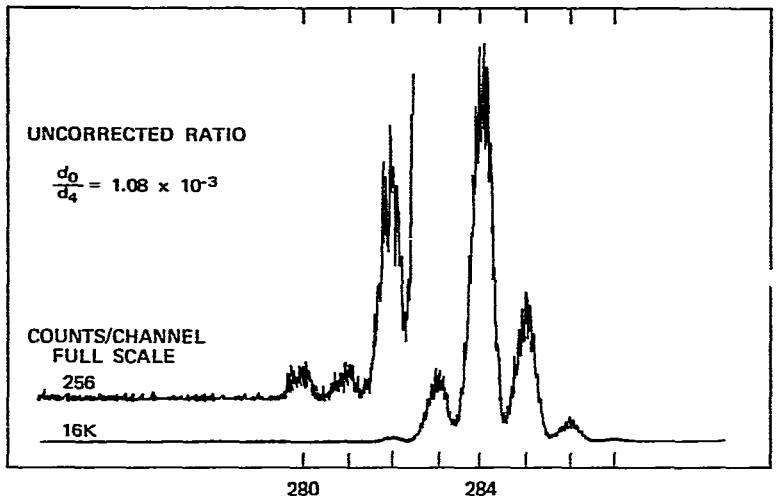


Fig. 1. Field ionization mass spectrum of ring-deuterated imipramine-d_4.

with water, extracted with methylene chloride, and 3-dimethylaminopropanol-d_6 was collected by distillation. The product was then treated with thionyl chloride in refluxing chloroform. The resulting N-dimethyl-3-chloropropylamine hydrochloride was precipitated by addition of toluene and was purified by recrystallization[18].

Iminodibenzyl-d_4 was reacted initially with sodium amide in dry benzene. The mixture was heated for 1 h to 78° and was then cooled to 40° and treated dropwise with N-dimethyl-3-chloropropylamine-d_6 in toluene. The resulting mixture was heated to reflux for 16 h, cooled, washed with water, and extracted into 2 *N* HCl[19].

The HCl extract was made basic with concentrated NaOH, and the suspension was extracted with diethyl ether. Removal of ether yielded the free base of imipramine-d_{10} in approximately 25% yield. The hydrochloride salt was reformed from an acetone solution of the base, and the product was recrystallized several times from acetone. Final purification was by silica gel TLC (chloroform–methanol–NH_4OH, 50:50:1).

The field ionization mass spectrum of the product is shown in Fig. 2. It is seen that the predominant mass actually occurs at *m/e* 289, corresponding to the d_9 isotopic variant, instead of *m/e* 290 which would be the d_{10}. This result suggests that slight back exchange of the ring deuterons occurred during the coupling of iminodibenzyl-d_4 with the N-dimethyl-3-chloropropylamine-d_6. The uncorrected isotopic ratios shown in Fig. 2 indicate, however, that very little unlabeled or tetradeuterated imipramine is present in the imipramine-d_{10}; thus, this material could serve as a carrier or isotopic dilutant for the two lower mass isotopic variants in stable isotope dilution analyses. This was the use for which the multilabeled imipramine was intended.

Gas chromatography–mass spectrometry

Structural analysis of the synthetic d_{10} material was performed using a computer-controlled LKB 9000 GC–MS system. To obtain the mass spectra, aqueous solutions of the imipramine salts were made basic and the suspensions were extracted

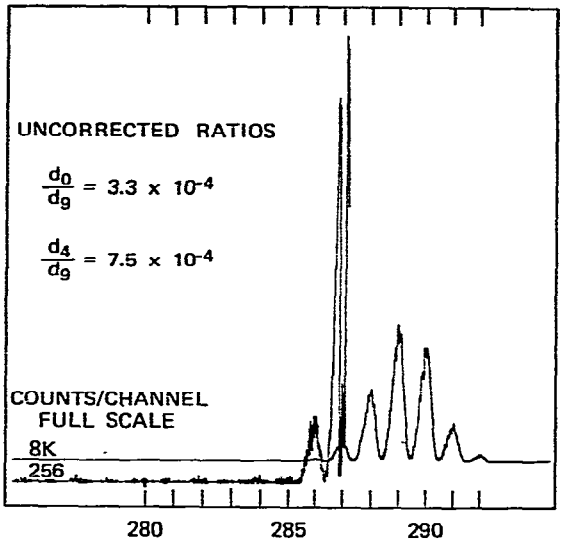


Fig. 2. Field ionization mass spectrum of multilabeled imipramine-d_{10}.

with 90% hexane–ether. Aliquots of the concentrated extracts were analyzed by GC–MS without prior derivatization.

The electron impact mass spectra of unlabeled imipramine and of the synthetic d_{10} material are shown in Fig. 3. In the region of the d_{10} molecular ions (*m/e* 290), it is seen that a cluster of peaks is present with the predominant mass being found at *m/e* 289. The same cluster was seen by field ionization mass spectrometry (Fig. 2). It is important to note that the spectrum shown at the bottom of Fig. 3 is unaltered by TLC under conditions that were subsequently shown to result in isotopic separations (see Results and discussion). Thus, TLC does not induce isotopic exchange nor does it produce a change in the molecular structure.

The fragmentation patterns of unlabeled imipramine and of the synthetic d_{10} material are very similar when the existence of two major labeled variants (*i.e.*, d_9 and d_{10}) is taken into consideration. Moreover, the cluster of peaks in the region of the molecular ion is mimicked in its essentials by the clusters found at *m/e* 197–198, 211–212, 223–224, and 238–239, but a similar pattern is not seen at *m/e* 64 and 91. This points to incomplete deuteration of the dibenzazepine moiety as the cause of the isotopic inhomogeneity, and supports the inference that back exchange of the ring deuterons took place during the coupling process.

Further evidence for the identity of the synthetic substance as imipramine-d_{10} was obtained by injecting the unlabeled compound together with the synthetic substance simultaneously into the gas chromatograph of the LKB 9000. The 5-ft. column (3% OV-1) was operated at a temperature of 290°. The resulting chromatogram showed only one peak for the two species injected. By recording mass spectra rapidly at different points on the peak, however, it was found that partial separation had occurred, with the d_{10} compound slightly preceding the d_0. Such a small separation can be ascribed to an isotope effect rather than to a nonisotopic structural difference. Numerous examples of isotopic fractionation in gas chromatography have been reported[1,3,9–11].

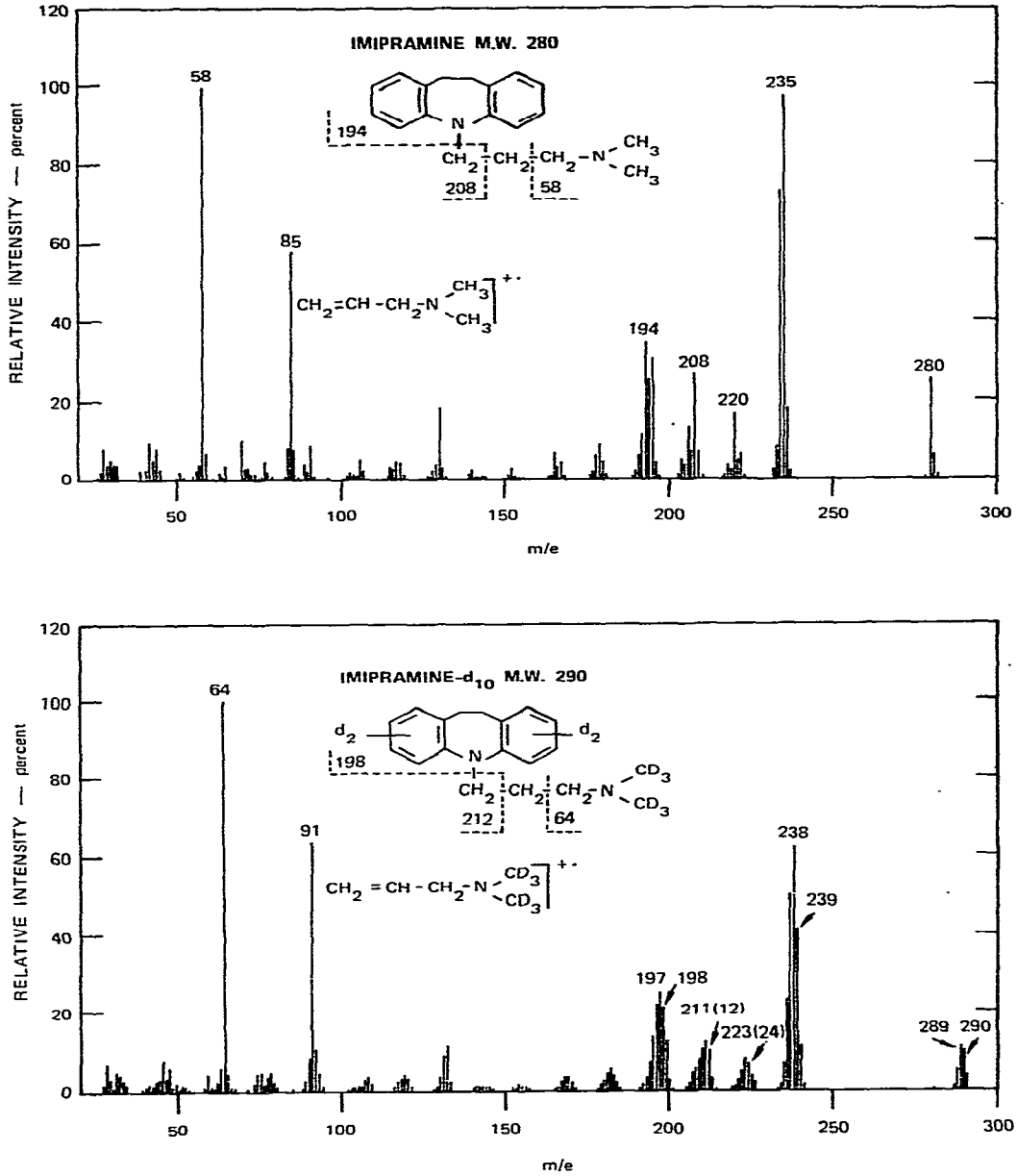


Fig. 3. Electron impact mass spectra of unlabeled imipramine (top) and multilabeled imipramine (bottom).

Proton magnetic resonance spectroscopy

Evidence that the synthetic d_{10} material is imipramine was also provided by proton magnetic resonance spectroscopy (Fig. 4). The spectra shown were obtained using a Varian EM-360 (60-MHz) spectrometer. Both spectra are of the imipramine-free base in acetone-d_6 with tetramethylsilane as reference.

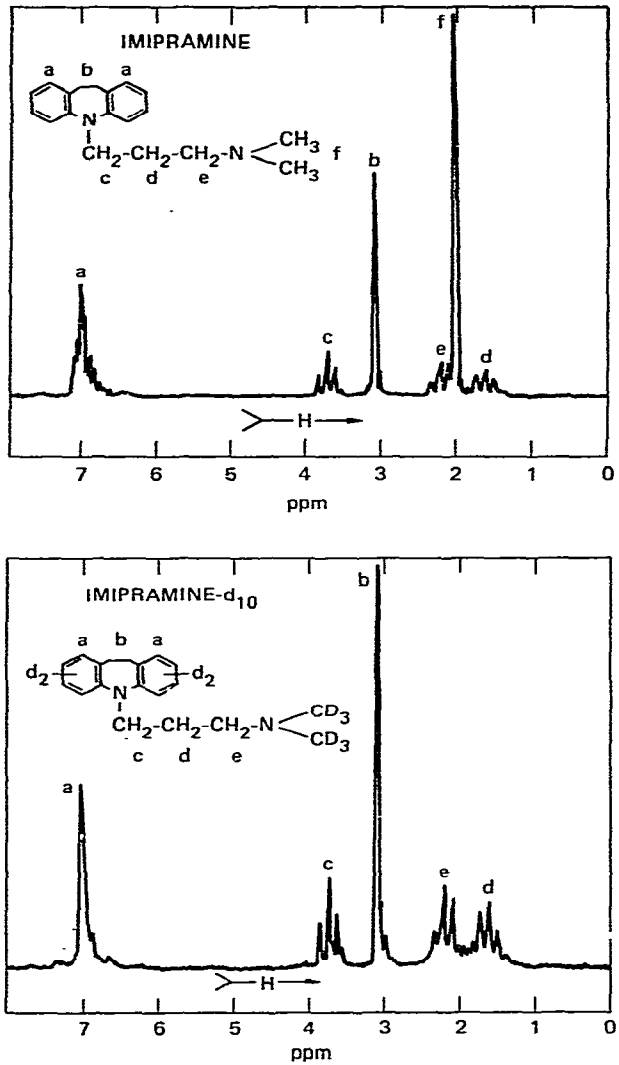


Fig. 4. Proton magnetic resonance spectra of unlabeled imipramine (top) and multilabeled imipramine (bottom) relative to tetramethylsilane.

Integration of the protons on the dibenzazepine moiety of imipramine-d_{10} accounts for four to five protons, as expected. The N-dimethyl protons detected in unlabeled imipramine at 2 ppm as a singlet (Fig. 4, top) are absent in the imipramine-d_{10} spectrum (Fig. 4, bottom). These spectra, together with the preceding mass spectrometric evidence, conclusively identify the synthetic material as imipramine-d_{10}. Proof of structure of the labeled compound was deemed necessary in view of the unprecedented isotopic separations that were observed (see Results and discussion).

Thin-layer chromatography

Thin-layer chromatographies of imipramine isotopic variants were carried out using glass plates coated with the appropriate adsorbent[20]. High-purity silica gel con-

taining a fluorescent indicator was a product of Macherey, Nagel & Co. (Düren, G.F.R.) (MN-Silica Gel G-HR/UV_{254}). The silica gel plates were activated by heating to 110° for 1 h before use.

Woelm alumina (basic) and a green fluorescent indicator (Woelm) were purchased from ICN (Cleveland, Ohio, U.S.A.). The plates were activated by heating to 130° for 30 min. Avicel microcrystalline cellulose was obtained from Brinkmann (Westbury, N.Y., U.S.A.). Cellulose-coated plates were heated briefly (105° for 10 min) before being used. The alumina and cellulose coatings contained 2% of the fluorescent indicator.

All chromatographies were carried out in tanks saturated with the appropriate solvent by means of filter paper moistened with the solvent and suspended on a wall. The solvent fronts ran the full length of the plates, at which time the plates were withdrawn. Imipramine spots were located by fluorescence quenching.

RESULTS AND DISCUSSION

Silica gel TLC of imipramine-d_{10} in the presence of unlabeled imipramine gave rise to two distinct spots. Typical thin-layer chromatograms of imipramine isotopic variants are shown in Fig. 5. The photograph clearly illustrates the remarkable resolutions achieved. Total separations between unlabeled imipramine and imipramine-d_{10} are seen in three of the four chromatograms. The one chromatogram that does not exhibit isotopic separation was developed with an acidic solvent.

The separations illustrated in Fig. 5 were obtained with a variety of solvents, as shown in Table I. The data of Table I can be briefly summarized by the following three statements: (1) partial or complete separations between d_0 and d_{10} imipramine were observed in all basic and neutral solvent systems; (2) in every case, the d_0 compound migrates more rapidly than the d_{10}; and (3) separations between d_0 and d_{10} imipramine were not observed in weakly acidic solvent systems.

These findings suggest that the chromatographic separations between the d_0 and d_{10} isotopic variants of imipramine are caused by differing proton affinities of the two compounds. Reports in the literature were found that lend strong support to this hypothesis. Van der Linde and Robertson[21] and Northcott and Robertson[22] have measured the basicities of perdeuterated methylamine, dimethylamine, and trimethylamine. In all cases, the deuterated amines were found to be significantly stronger bases than the corresponding protiated compounds (*e.g.*, for dimethylamine, $K_D/K_H = 1.31$). It should be emphasized that the effects observed are secondary isotope effects resulting from deuterium substitution in the methyl group(s) on a protonic equilibrium.

From the data of Van der Linde and Robertson[21] and Northcott and Robertson[22], we would expect that imipramine-d_{10} would be a stronger base than unlabeled imipramine, and would interact more strongly with the weakly acidic Si–OH groups ($pK_a \approx 6$–8)[23] of silica gel. The observed result that imipramine-d_{10} always migrates more slowly than unlabeled imipramine in basic and neutral solvents is consistent with this hypothesis. Since isotopic fractionation is not found under weakly acidic conditions, we may infer that interactions between the tertiary amine and the Si–OH groups are of negligible importance in comparison to the interactions between the amine and the solvent.

Isotopic fractionation is not caused by the deuterium atoms of the dibenzazepine moiety of the compound. This was proven by chromatographing unlabeled

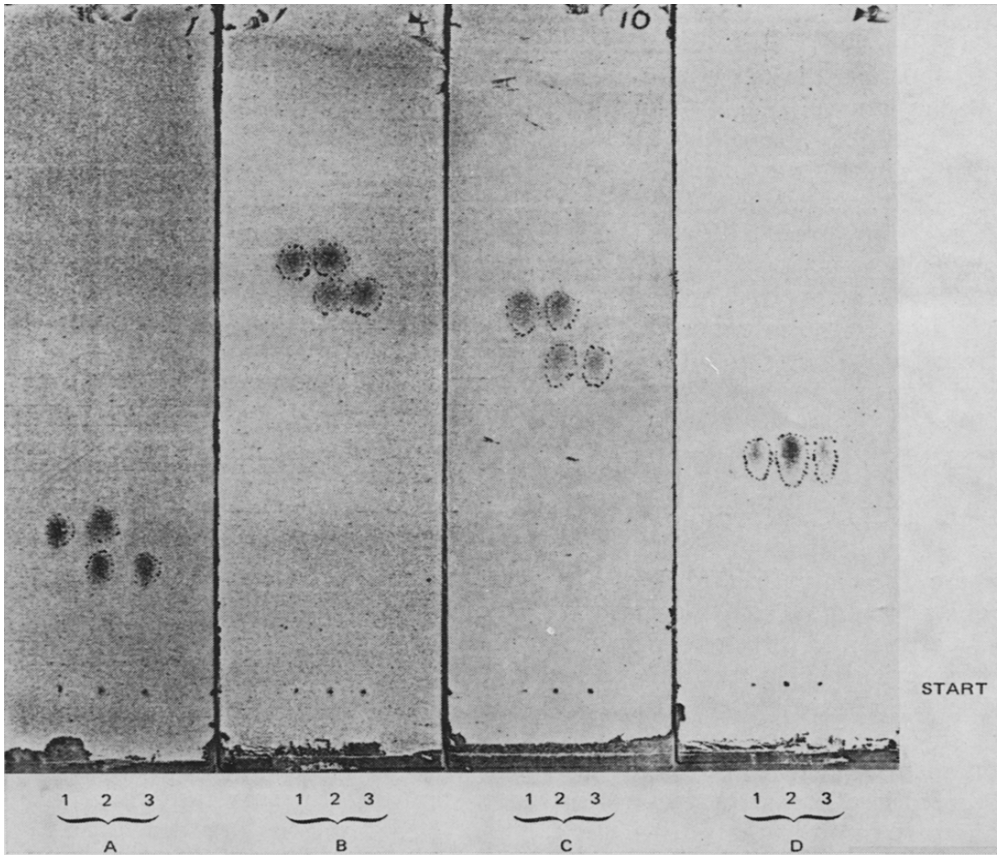


Fig. 5. Silica gel thin-layer chromatograms of imipramine isotopic variants. Solvents: (A) benzene–acetone–NH_4OH (300:60:1); (B) methanol–NH_4OH (200:3); (C) chloroform–methanol (4:1); (D) chloroform–methanol–glacial acetic acid (50:50:1). Visualization followed exposure to iodine vapor. 1 = Unlabeled imipramine; 2 = unlabeled imipramine + imipramine-d_{10}; 3 = imipramine-d_{10}.

imipramine and imipramine-d_4 under conditions that lead to complete separation between unlabeled imipramine and imipramine-d_{10}. There was no detectable separation between unlabeled imipramine and imipramine-d_4 under these conditions. The observed fractionation is solely caused by deuterium substitution in the methyl groups of the dimethylaminopropyl side chain of the compound.

Other TLC adsorbents besides silica gel were tested briefly to assess whether isotopic fractionation occurs with those adsorbents as well. Adsorption chromatography on Woelm alumina (basic) produced isotopic separations similar to those observed with silica (Table II). Partition chromatography on cellulose, however, did not result in isotopic fractionation in either basic or acidic solvents (Table II).

The isotope effects found with the inorganic adsorbents and the absence of an effect in cellulose chromatography are consistent with the above interpretation that acid–base interactions between the imipramine molecule and the adsorbent are responsible for the fractionation effect. Cellulose is an extremely weak acid, so that such interactions are relatively unimportant in a highly protic solvent such as methanol–water. Both silica and alumina, however, possess weakly acidic groups that can func-

TABLE I

SILICA GEL THIN-LAYER CHROMATOGRAPHY OF IMIPRAMINE ISOTOPIC VARIANTS

All chromatographies were performed in tanks saturated with the appropriate solvent by means of filter paper moistened with the solvent and suspended on a wall.

Solvent system	R_F	
	d_0	d_{10}
Basic		
Benzene–acetone–NH_4OH (300:60:1)	0.24	0.18
Benzene–dioxane–methanol–NH_4OH (100:80:10:1)	0.60	0.52
Methanol–NH_4OH (200:3)	0.62	0.57
Acetone–NH_4OH (100:1)	0.65	0.58
Chloroform–methanol–NH_4OH (50:50:1)	0.75	0.71
Dioxane–chloroform–acetone–NH_4OH (95:90:10:5)	0.76	0.73
Benzene–ethyl acetate–methanol–NH_4OH (75:75:15:2)	0.84	0.79
Benzene–methanol–NH_4OH (133:21:2)	0.89	0.88
Neutral		
Chloroform–methanol (1:1)	0.33	0.27
Chloroform–methanol (4:1)	0.54	0.47
Chloroform–methanol–water (4:2:1) (lower phase, plus 5% methanol)	0.69	0.65
Acidic		
Benzene–methanol–acetic acid (50:50:1)	0.32	0.32
Chloroform–methanol–acetic acid (50:50:1)	0.33	0.33
Ethyl acetate–methanol–acetic acid (45:255:2)	0.33	0.33
Methanol–acetic acid (150:1)	0.38	0.38

TABLE II

THIN-LAYER CHROMATOGRAPHY OF IMIPRAMINE ISOTOPIC VARIANTS ON ALUMINA AND CELLULOSE

Adsorbent	*Solvent system*	R_F	
		d_0	d_{10}
Woelm alumina (basic)	Methanol	0.69	0.65
	Chloroform–isopropanol (1:1)	0.87	0.85
Avicel microcrystalline cellulose	Methanol–water–NH_4OH (100:45:1)	0.92	0.92
	Methanol–water–acetic acid (60:90:1)	0.85	0.85

tion as proton donors in basic or neutral solvents. In sufficiently acidic solvents, where the adsorbent is a much less effective proton donor than the solvent itself, isotopic fractionation does not occur.

Implications of the isotope effect

The isotopic fractionation found in TLC suggests a cautionary approach to the deuterium labeling of molecules for isotope dilution analyses. The evidence of this paper shows that secondary isotope effects can profoundly influence the chromatographic properties of molecules. Deuterium labeling in the proximity of acidic or basic functional groups should be undertaken with reservations, particularly when chromatography on silica gel is used for purification. The type of chromatography, whether TLC or high-pressure liquid chromatography, is less important than the type of adsorbent and the location of the label in producing an isotope effect.

To avoid the occurrence of isotopic fractionation in the TLC of imipramine, we have synthesized other isotopic variants of the compound that do not incorporate deuterium in the side chain. Besides imipramine-d_4, imipramine-d_2 has been prepared (labeled at C_{10} and C_{11}), as well as imipramine-d_6. None of these exhibit detectable isotopic fractionation in TLC. The details of these syntheses will be described elsewhere.

Isotopic fractionation has been observed frequently using other chromatographic techniques[1–14]. On-line GC–MS systems that do not integrate over total peak areas but merely measure an instantaneous isotopic ratio at some position on the peak (usually the maximum) may be particularly susceptible to error caused by isotopic fractionation. (For example, in the present case, the imipramine-d_{10} peak emerged before that of imipramine-d_0 in gas–liquid chromatography.) The preparation of a standard calibration curve is an effective means of discovering such effects when they occur.

ACKNOWLEDGEMENTS

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