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Note

Regiochemical differences in the isotopic fractionation of deuterated benzoic acid isotopomers by reversed-phase high-performance liquid chromatography

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The isotopic fractionation of mixtures of perdeuterated and unlabelled organic compounds by reversed-phase liquid chromatography is well established. Generally the perdeuterated isotopomers exhibit less retention than their unlabelled analogues. Various explanations have been advanced to account for this behaviour[1–5]. However, most studies have failed to investigate regiochemical or structural contributions to the observed chromatographic isotope effects. This is surprising since structural parameters are known to be dominant in the isotopic fractionation of many organic compounds on silica chromatography. The most widely reported examples of these latter structural effects are ascribed to changes in the base strength of organic amines bearing isotopically labelled methyl or methylene groupings[5–8]. Given this experience with normal-phase chromatography, studies of structural effects upon reversed-phase isotopic fractionation would seem prudent before detailed theoretical explanations of retention behaviour are proposed.

Over the last few years a number of variously substituted isotopomers of benzoic acid have been synthesised in our laboratories as a result of our investigations into exchange-labelling techniques[9,10]. The availability of these isotopomers has provided the opportunity to study regiochemical effects in the isotopic fractionation of benzoic acids by reversed-phase high performance liquid chromatography (HPLC). The results of these studies are described herein.

MATERIALS AND METHODS

Synthesis of isotopomers

Pentadeuterobenzoic acid was prepared by a modification of the platinum-catalysed exchange procedure developed by Garnett and Brown[11] as previously described[10].

3,4,5-Trideuterobenzoic acid was prepared both by a palladium-catalysed exchange procedure[12] and also by dedeuteration of pentadeuterobenzoic acid in the presence of rhodium(III) chloride and protiated water[10].

2,3,5-Trideuterobenzoic acid, 4-deuterobenzoic acid, 3-deuterobenzoic acid and 2-deuterobenzoic acid were prepared by deuterodehalogenation of 2,3,5-triiodo-

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benzoic acid, 4-bromobenzoic acid, 3-bromobenzoic acid and 2-iodobenzoic acid, respectively, using the Raney copper alloy deuterodehalogenation procedure developed by Tashiro *et al.*[13].

3,5-Dideuterobenzoic acid, 2,4-dideuterobenzoic acid, 3,4-dideuterobenzoic acid and 2,5-dideuterobenzoic acid were prepared by reductive dehalogenation, in deuterated methanol, of the corresponding dichlorobenzoic acids using dideuterium gas and a 5% palladium on carbon catalyst.

2,6-Dideuterobenzoic acid was prepared by rhodium(III) chloride-catalysed isotope exchange between unlabelled benzoic acid and deuterium oxide[9].

Chromatography

HPLC conditions. Samples were chromatographed using two 250 × 4.9 mm Hypersil ODS columns linked in series. The mobile phase used was methanol–water (3:7, v/v) to which 1% (v/v) formic acid had been added. This mobile phase was delivered at a combined flow-rate of 1.0 cm^3 min^{-1} by both channels of a Milton Roy duplex pumping system. Detection was carried out using a Pye Unicam 4020 ultraviolet absorbance detector set to monitor at 280 nm and 0.32 a.u.f.s. Injection volumes of 300 mm^3 were used throughout the analyses.

Preparation of samples for injection. Solutions for analysis were prepared by mixing solutions, prepared in the chromatographic mobile phase, of the labelled benzoic acid (1.0 cm^3, 0.1 mg cm^{-3}), unlabelled benzoic acid (1.0 cm^3, 0.1 mg cm^{-3}), *p*-nitrobenzoic acid (0.2 cm^3, 0.1 mg cm^{-3}) and *p*-hydroxybenzoic acid (0.1 cm^3, 0.1 mg cm^{-3}).

Processing of data

Data processing was carried out in the same fashion for all the chromatograms. First, the half-height peak width ($w_{0.5}$) of the combined peak arising from the deuterated and unlabelled isotopomers was measured. Second, this measured width was corrected for run-to-run variations in retention by taking the ratio of $w_{0.5}$ to the measured retention of the *p*-nitrobenzoic acid internal standard. This ratio was measured on at least four separate occasions for each of the isotopomers. All the ratios determined for the isotopomers were then normalised by dividing each by the corresponding mean ratio determined for unlabelled benzoic acid. The resulting normalised mean ratios, which serve as a measure of the isotopic fractionation, are shown in Table I. The table also includes corresponding data in which the isotopic fractionation, expressed as above, has been corrected for the number of deuterium atoms present in each of the isotopomers. In addition, the table contains measured retention data for those isotopomers in which resolution of the labelled from the unlabelled compound was observable as distinct peak-splitting rather than mere broadening of the combined peak. These latter data are expressed as the ratio of the retention time of the unlabelled benzoic acid to the retention time of the deuterated isotopomer.

RESULTS AND DISCUSSION

Selection of chromatographic conditions

Since it was desirable to study the chromatographic fractionation of the isotopomers in as simple a system as possible, the choice of chromatographic conditions

TABLE I

FRACTIONATION OF BENZOIC ACID ISOTOPOMERS

Isotopomer	*Mean ratio ± S.D.*	*Mean ratio per ^2H atom*	*Retention ratio ($H/^2H$)*
2,3,4,5,6-^{2}H	3.61 ± 0.09	0.72	1.038
3,4,5-^{2}H	3.00 ± 0.08	1.00	1.029
2,3,5-^{2}H	2.61 ± 0.03	0.87	1.023
3,4-^{2}H	2.40 ± 0.03	1.20	1.019
3,5-^{2}H	2.31 ± 0.07	1.16	1.019
2,4-^{2}H	2.02 ± 0.05	1.01	1.013
2,5-^{2}H	1.93 ± 0.05	0.96	1.010
2,6-^{2}H	1.39 ± 0.03	0.70	NR
4-^{2}H	1.53 ± 0.06	1.53	NR
3-^{2}H	1.41 ± 0.07	1.41	NR
2-^{2}H	1.13 ± 0.04	1.13	NR
0-^{2}H	1.00 ± 0.04	–	–

was restricted to those likely to yield reversed-phase partition as the major chromatographic mode, i.e. simple octadecyl stationary phases with mobile phases consisting of a mixture of water and an organic modifier.

To ensure chromatography as a single (unionised) species the pH of the mobile phase was adjusted by the addition of 1% (v/v) of formic acid to suppress ionisation of the carboxyl group. This quantity of formic acid was quite sufficient to ensure the desired symmetrical peak shape. Moreover, the addition of a stronger acid (trifluoroacetic acid) produced no difference in peak shape or in isotopic fractionation.

Initially, 5-μm Spherisorb C_{18} was investigated as the stationary phase. Subsequently, however, a higher degree of isotopic fractionation was observed with the more highly capped Hypersil ODS. Since this latter stationary phase also yielded more efficient chromatography (*i.e.*, higher plate numbers) it was selected for all the quantitative studies reported below.

Following selection of the above chromatography conditions, the retention of unlabelled benzoic acid was adjusted by selecting a suitable organic modifier concentration. A methanol concentration (30%) which yielded optimum peak shape and efficiency at a final capacity factor (k') of around 8.4 was selected.

Fractionation measurements

To study the isotope effect associated with each isotopomer the variously deuterated benzoic acids were each chromatographed as a 1:1 mixture with unlabelled benzoic acid. Under these conditions isotopic fractionation of the deuterated isotopomer from unlabelled benzoic acid was observed as a broadening or splitting of the combined peak. Internal standards with retentions shorter (*p*-hydroxybenzoic acid) and longer (*p*-nitrobenzoic acid) than benzoic acid were included in all the runs to check for any deterioration in column resolution. The latter standard was also used to allow correction for any small day-to-day variations in retention.

Although isotopic fractionation was readily observed for most of the deuterated substrates using a single 250 × 4.9 mm Hypersil ODS column, the studies de-

scribed employed two such columns linked in series. This provided a larger degree of isotopic fractionation and consequently yielded improved accuracy in the measurement of half-heights and peak widths for the partially resolved peaks. Under these conditions the combined columns had an efficiency of 21 800 and 22 200 plates measured for benzoic acid ($k' = 8.36$) and *p*-nitrobenzoic acid ($k' = 9.05$) respectively.

The quantitative results are shown in Table I and Fig. 1, whilst Fig. 2 shows some typical examples of the chromatograms obtained. Taken over all, the results demonstrate the expected decrease in retention with increasing extent of deuterium substitution. However, this decrease proves to be highly dependent upon the regiochemistry of the deuterium substitution.

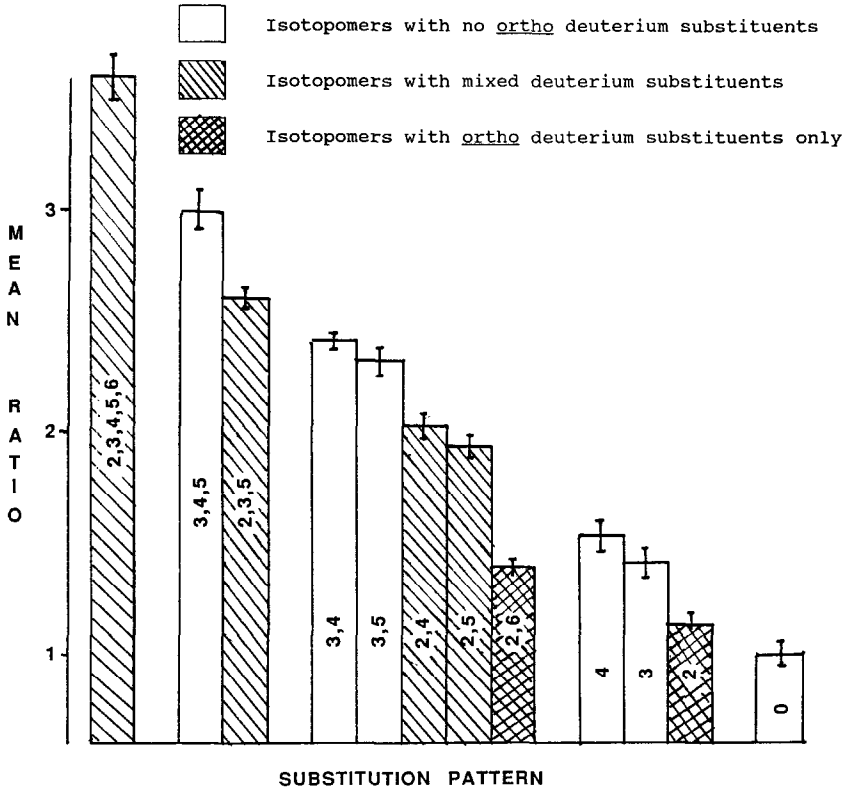

Fig. 1. Fractionation of benzoic acid isotopomers *vs.* substitution pattern. The numbers in the columns indicate the ^2H substitution positions.

Fig. 1 shows the observed isotopic fractionation in histogram form. It is noteworthy that although the isotopomers can be grouped according to the number of deuterium substituents, nevertheless, within each group, the fractionation shows clear variations dependent upon the actual substitution pattern. In particular, those isotopomers which contain deuterium substitution at either the 2 or the 6 (*ortho*) positions all display substantially lower isotopic fractionation than equally deuterated

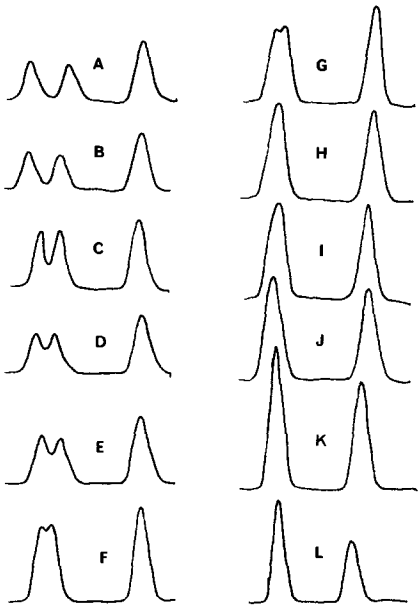

Fig. 2. Chromatographic fractionation of benzoic acid isotopomers. Typical examples of chromatograms from mixtures of variously deuterated benzoic acids and unlabelled benzoic acid. The peak at the right of the chromatograms is the *p*-nitrobenzoic acid internal standard. For clarity only the region at $k' > ca.$ 8 is shown. The isotopomer substitution patters are: (A) 2,3,4,5,6-^{2}H; (B) 3,4,5-^{2}H; (C) 2,3,5-^{2}H; (D) 3,4-^{2}H; (E) 3,5-^{2}H; (F) 2,4-^{2}H; (G) 2,5-^{2}H; (H) 2,6-^{2}H; (I) 4-^{2}H; (J) 3-^{2}H; (K) 2-^{2}H; (L) unlabelled benzoic acid.

isotopomers in which the deuterium substituents are restricted to the 3, 5 and 4 (*meta* and *para*) positions. Moreover, the isotopic fractionation of 2,6-dideuterobenzoic acid, which possesses two *ortho* deuterium substituents, is much less than for all the other dideuterated spieces. Similarly, the data in Table I shows the expected overall increase in fractionation with increasing degree of substitution. However, when the isotopic fractionation is calculated per deuterium atom substituted, it is clear that those isotopomers containing *ortho* deuterium substituents display lower isotopic fractionation than their *meta*/*para* deuterated counterparts.

Overall it appears that the chromatographic isotope effect resulting from *ortho* substitution with deuterium is considerably less than for *meta* or *para* substitution.

The above behaviour could result from a number of structural effects, either steric or electronic in origin. However, since the fractionation is occurring under conditions remote from the pK_a of benzoic acid, it is unlikely that isotope effects upon the acidity of the carboxyl group are responsible for the behaviour. Moreover, previous studies of the exchange deuteration of variously substituted aromatics[14,15] and, more recently, of unionised benzoic acid itself[12] have demonstrated considerable '*ortho* deactivation' by bulky substituents. Since this process also involves the interaction of the benzoic acid molecule with a solid surface, there is some precedent for ascribing the observed chromatographic behaviour largely to steric shielding of the *ortho* positions by the carboxyl group. This would prevent any *ortho* deuterium substituents from exerting their maximal isotope effect upon retention. If this is true then

interaction of the *meta* and *para* area of the molecule with the stationary phase plays a major role in the retention process in this case. Thus, isotopic effects associated with the *ortho* positions are minimised, either because the change in molecular volume[3,4] due to isotopic substitution is taking place in a volume of the molecule which is already shielded by a voluminous group, or because the Van der Waals interactions between *ortho* C–H or C–^{2}H bonds and the hydrocarbon residues of the stationary phase[1,2] are minimised by the steric bulk of the carboxyl group.

CONCLUSIONS

Variously deuterated isotopomers of benzoic acid can be separated from unlabelled benzoic acid to degrees dictated by both the extent of isotopic substitution, and by the position of deuterium substitutions within the molecule. The results reported clearly demonstrate that, although isotopic fractionation does indeed increase with degree of substitution, regiochemical factors can introduce significant quantitative deviations from a simple linear or additive model of the isotope effect.

Since significant regiochemical variations in isotope effects can be demonstrated in such a simple reversed-phase chromatographic system, considerable attention to regiochemical factors is clearly needed in the design of studies to investigate changes in molecular properties, *e.g.*, lipophilicity, caused by isotopic substitution.

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