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Studies on an abnormally sharpened elution peak observed in counter-current chromatography

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ABSTRACT

Counter-current chromatography (CCC) of the bromoacetylation product of 3,3',5-triiodo-L-thyronine (T_3) produced an unusually sharp peak for the desired product, N-bromoacetyl T_3 ($BrAcT_3$). A series of experiments revealed that bromoacetic acid, probably present as a side reaction product in the sample solution, was responsible. This compound repressed the ionization of the carboxyl group of $BrAcT_3$ forcing it into the less polar stationary phase until the bromoacetic acid had eluted completely from the apparatus. At this point, the sudden increase of pH and consequent ionization of the $BrAcT_3$ allowed the ammonium salt of the latter to enter the more polar moving phase where it eluted rapidly from the column as a sharp peak. The same phenomenon was observed in the CCC fractionation of a series of indole auxins where addition of trifluoroacetic acid to the sample caused peak sharpening by the same process. The phenomenon recalls pH gradient elution and isoelectric focussing except that the substance responsible for the pH range here is added along with the sample in one bolus forming a sharp pH gradient at its trailing edge. As with gradient elution, the technique is of practical interest since it permits collection of the eluting compounds with increased detectability in fewer fractions. The technique can also enhance separation of compounds whose partition coefficients differ with a change in pH.

INTRODUCTION

Counter-current chromatography (CCC) is a continuous chromatographic process [1,2] depending on counter-current partitioning of two liquid phases [3]. The partitioning action takes place in an open column space free of the support matrix found in ordinary liquid chromatography systems. Because of this, the adsorption and peak tailing sometimes encountered in liquid chromatography is absent and the CCC method is expected to provide symmetrical peaks as long as the partitioning phases are not overloaded with sample. As with other chromatographic systems using isocratic elution, peak width increases with retention time due to distribution and diffusion or when more than one species is present as in the case of ionization of a carboxylic acid.

During the CCC purification of a synthetic sample of N-bromoacetyl-3,3',5-triiodo-L-thyronine ($BrAcT_3$), we encountered an unexpectedly sharp elution peak (Fig. 1A) for this substance [4]. The present note describes a series of experiments which revealed the agent responsible for the peak sharpening to be bromoacetic acid, probably a side reaction product, that had been introduced with the sample. A mechanism for the effect is proposed and

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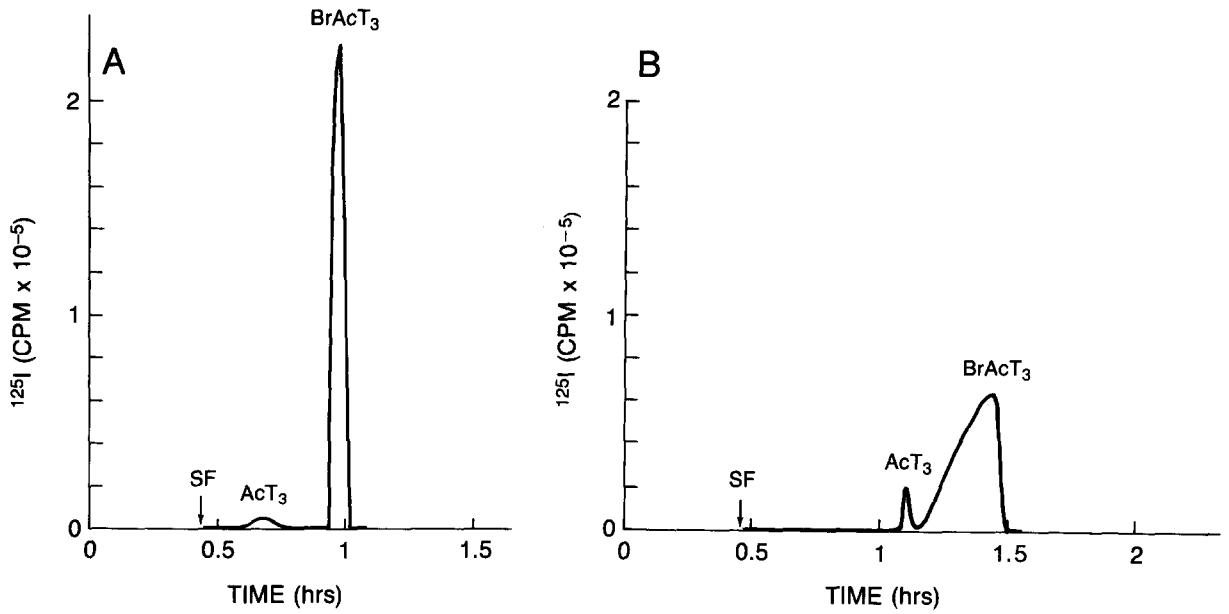


Fig. 1. Chromatograms of $BrAcT_3$ obtained with a standard two-phase solvent system composed of hexane, ethyl acetate, methanol and 15 m*M* ammonium acetate (pH 4) at two different volume ratios, (A) 1:1:1:1 and (B) 4:5:4:5, but otherwise identical experimental conditions. Other experimental conditions are as follows: sample: crude bromoacetylation mixture (from 0.1 mmol T_3) and a minute amount of [^{125}I]T_3 in 4 ml of solvent consisting of equal volumes of upper and lower phases; mobile phase: lower aqueous phase (pH 5.2); flow-rate: 3 ml/min; speed: 800 rpm; detection: ^{125}I radioactivity; retention of stationary phase: 69.8% (A) and 64.7% (B). SF = Solvent front. Note that the peak profile of $BrAcT_3$ and AcT_3 was reversed by changing the solvent ratio.

extension of the technique to other eluates and peak sharpening agents is demonstrated.

EXPERIMENTAL

All separations were performed with a commercial model of the high-speed CCC centrifuge (Ito Multilayer Separator/Extractor from P.C. Inc., Potomac, MD, USA). The design of the apparatus has been reported earlier [4].

The two-phase solvent system was prepared by mixing hexane, ethyl acetate, methanol and an aqueous solution of 15 m*M* ammonium acetate at a volume ratio of 1:1:1:1, where the pH of the ammonium acetate solution was adjusted by adding acetic acid. The solvent mixture was thoroughly equilibrated at room temperature in a separatory funnel and the two phases were separated shortly before use.

In each separation, the entire column was first filled with the stationary non-aqueous phase followed by injection of 2 to 4 ml of sample solution containing crude $BrAcT_3$ [4] or a mixture of indole auxins through the sample port. The mobile aqueous phase was then pumped into the head of the column at a flow-rate of 3 ml/min while the apparatus was spun at 800 rpm. The effluent from the outlet of the column was continuously monitored with a UV monitor (Uvicord S; LKB, Bromma Stockholm, Sweden) at 280 nm and collected in glass tubes to obtain 3-ml fractions. After all desired peaks had been eluted, the run was terminated and the column contents were collected in a graduated cylinder to determine the volume of the stationary phase retained in the column.

In the separation of radioactive samples, the radioactivity of each fraction was measured with a gamma scintillation counter (Auto-Gamma 500 Series; Packard Instruments, Downers, IL, USA). The pH of each fraction was also determined by a portable pH meter (Accumet Portable Laboratory; Fisher Scientific, Pittsburgh, PA, USA).

RESULTS AND DISCUSSION

Fig. 1A illustrates the sharp $BrAcT_3$ peak mentioned earlier [4]. To examine the effect of the solvent system on the elution of $BrAcT_3$, the mixture was reexamined using a slightly more polar solvent system. As expected, both the $BrAcT_3$ and the by-product eluted later due to the increased polarity of the solvent (Fig. 1B), however the sharpening effect was reversed. More importantly, merely diluting the original sample of $BrAcT_3$ resulted in a chromatogram in which both peaks were of normal shape. These facts suggested that a substance was present in the original sample that, though invisible to the detector, was strongly affecting the partitioning action. Mass spectrometric analysis showed that the original sample contained much bromoacetic acid. To prove the point, a solution of 2.5 mmol of bromoacetic acid was deliberately added to an amount of prepurified $BrAcT_3$ corresponding to that seen in Fig. 1A and monitored by pH as it eluted (Fig. 2, dotted line). Because of the quantity used, the stationary phase is considerably overloaded and it elutes with a sharply defined trailing edge just before the sharp peak of $BrAcT_3$ (Fig. 2, solid line). Clearly, the bromoacetic acid has acted to delay elution of the $BrAcT_3$ by repressing its ionization and forcing it to partition more completely into the non-polar stationary phase.

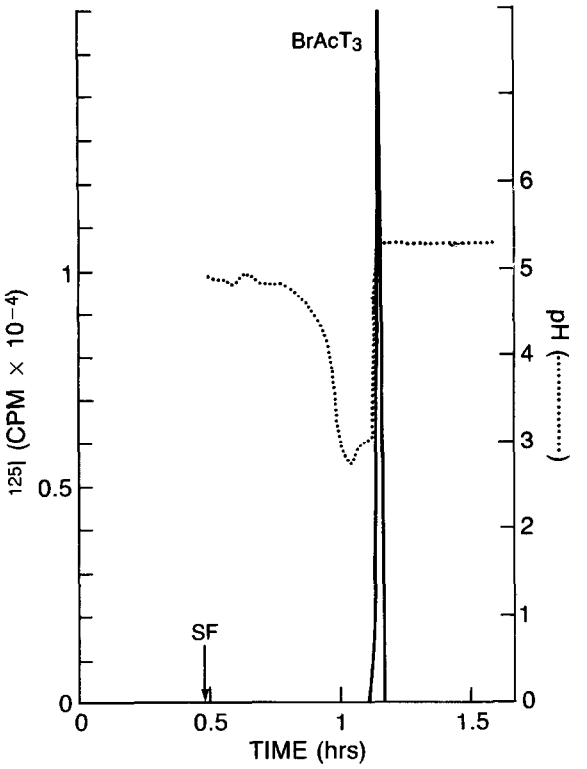
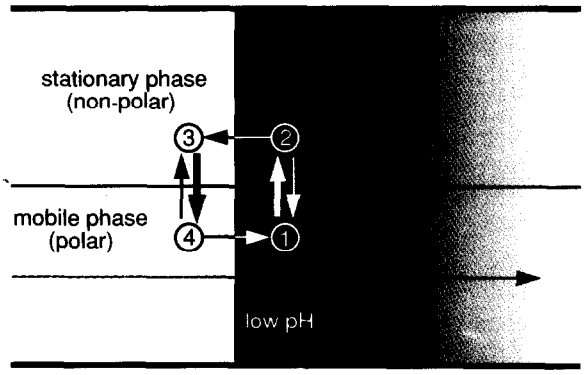


Fig. 2. Chromatogram of prepurified $BrAcT_3$ obtained with 2.5 mmol bromoacetic acid in the sample solution. A sharp peak profile of $BrAcT_3$ in Fig. 1A was reproduced by adding bromoacetic acid in the sample solution. Close association between the abrupt return point of pH curve and the sharp $BrAcT_3$ peak strongly suggests that bromoacetic acid is the causative agent of the sharp peak. Sample solution: CCC-purified $BrAcT_3$ (*ca.* 0.04 mmol) with bromoacetic acid (2.5 mmol) in 3.5 ml lower phase. Other experimental conditions as in Fig. 1A. Retention of the stationary phase was 67%. SF = Solvent front.

The above peak sharpening effect can be seen in detail in Fig. 3A where a schematic view of the column is shown with the stationary and moving phases arbitrarily separated. Thus, $BrAcT_3$ anions, that happen to be at location 1 in the presence of bromoacetic acid will rapidly protonate due to the lower pK_a of the excess bromoacetic acid and enter the nonpolar stationary phase (location 2). As the sharply defined trailing edge moves forward, the compound finds itself in location 3 where the stationary phase is in contact with the higher pH mobile phase. Ionization of the carboxylic acid occurs and the $BrAcT_3$ enters the mobile phase (location 4) whereupon it rapidly migrates to location 1 and the process is repeated. Thus, although elution of the sample is delayed, it undergoes the same partitioning action with distribution and diffusion lessened by the focussing action of the bromoacetic acid edge. Clearly, for the focussing action to take place there must be a particular relationship between the partition coefficients of the sample and causative agent. Fig. 3B details further the general requirements for the effect to be manifest. Thus when partition coefficients $[C_{s(\text{tationary})}/C_{m(\text{obile})}]$ of both the free acid (K_a) and its salt (K_b) are less than the partition coefficient of the causative acid (K_{pH}), the solute will elute earlier (peak 1) than the pH gradient and there will be no sharpening. On the other hand, when both K_a and K_b are greater than K_{pH}, the solute will elute after the gradient (peak 4), again without sharpening. Sharpening occurs only when K_{pH} falls between K_a and K_b as shown in peaks 2 and 3.

At first glance, the focussing effect recalls ordinary gradient elution [5] used extensively to reduce



PARTITION COEFFICIENT
($K = C_s/C_m$)

$K_b < K_a < K_{pH}$	Peak 1
$K_b << K_{pH} < K_a$	Peak 2
$K_b < K_{pH} << K_a$	Peak 3
$K_{pH} < K_b < K_a$	Peak 4

K_{pH} : acid agent
K_a : sample in low pH
K_b : sample in high pH

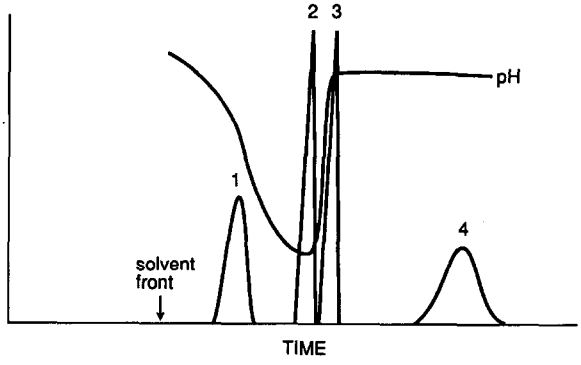


Fig. 3. (A) Schematic illustration of the peak sharpening process in the separation column. A portion of the column contains the non-polar stationary phase in the upper half and the polar mobile phase in the lower half where the solute molecules circulate at the sharp edge of the low pH region (shaded). For more detailed description, see the Results and Discussion section. (B) General requirements for sharp peak formation. Peak 1 is obtained when both K_a and K_b are smaller than K_{pH} while peak 4 is obtained when both K_a and K_b are greater than K_{pH}. Sharp peaks 2 and 3 are formed when K_{pH} falls between K_a and K_b as indicated above.

elution times and sharpen peaks in column chromatography of amino acids [6] and counter-current separation of alkaloids [7,8] where the specific relations between partition coefficients and ionization constants are discussed. Another apparently related process is isoelectric focusing [9] where an eluting ampholyte is trapped at a point in an established gradient where its effective charge is zero. However, in the present case, the shape of the pH gradient is unique since it is caused by the addition of the acid in one bolus along with the sample. Its effect on the eluting sample is therefore determined both by its quantity and its specific elution characteristics in the solvent system employed.

Inorganic acids including HCl and HBr elute rapidly due to their high polarity and, as expected from the above analysis, we have found that they do not sharpen the $BrAcT_3$ peak. However, trifluoroacetic acid (TFA), selected as a more convenient pH gradient-forming agent because of its easy removal

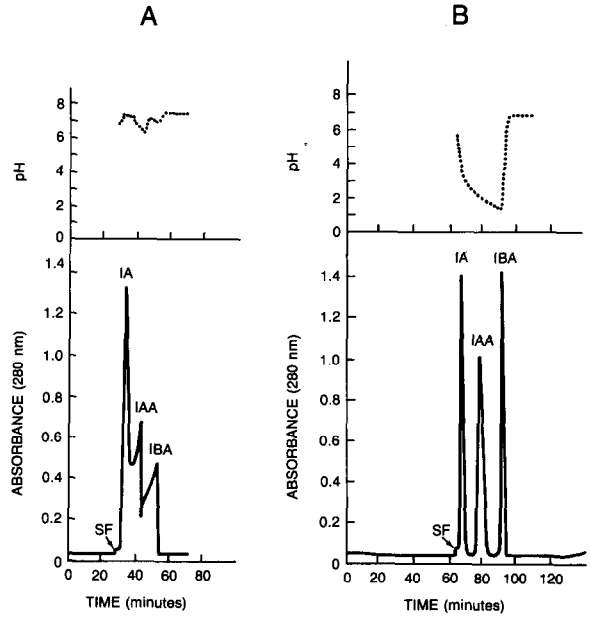


Fig. 4. Chromatograms of indole auxins obtained with a neutral solvent system using sample mixtures containing 2 μl TFA (A) and 400 μl TFA (B). Note that the amount of TFA in the sample solution determines the retention volume of the abrupt return point of the effluent pH which coincides with the sharp peak of one of the auxins. Solvent system: hexane–ethyl acetate–methanol–15 m*M* ammonium acetate, pH 7 (1:1:1:1); sample: indole auxins, IA, IAA and IBA (2 mg each) + 2 μl TFA (A) and 400 μl TFA (B) in 2 ml of solvent (1 ml of each phase); mobile phase: lower aqueous phase (pH 7.4); flow-rate: 3 ml/min; speed: 800 rpm. Retention of the stationary phase was 66.8% (A) and 28.1% (B). The low stationary phase retention in B is apparently due to introduction of a large volume of TFA in the sample solution. SF = Solvent front.

from the fractions (b.p. 72.4°C), served equally well for the chromatography of $BrAcT_3$.

To illustrate the generality of the technique, a set of indole auxins, indole-3 acetamide (IA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), was selected as a further example since these compounds have suitable partition coefficients in the above two-phase solvent system. With a trace of TFA added to the sample, the mixture at this pH produces broad and skewed peaks at short elution times as expected for dissociating substances (Fig. 4A). Addition of 400 μl of TFA results in a considerable shift of the pH edge toward longer times (Fig. 4B) to coincide with the elution of the IBA which is now sharpened. Under these conditions, the IAA peak is now beginning to return to the normal profile.

CONCLUSIONS

Since this peak sharpening phenomenon is dependent only on the partioning process, the present technique should be generally useful in liquid chromatography of ionizable substance wherever it is necessary to concentrate solutions in as few fractions as possible for enhanced detection or simplification of workup. The method should work as well for basic compounds by using a weak base to establish the pH profile. Of course, successful sharpening can be brought about only by careful consideration of the partition coefficients involved and these are not always easy to obtain particularly with unknown compounds.

An important feature of the method is its ability to cause a change in the *relative* elution times of components. This may be of use where impurities coelute with the desired analyte. Separation can be brought about as long as the impurities do not respond to a pH change in similar fashion. Thus, the technique is effective in shifting organic acids and bases away from neutral compounds.

Recently we have found that the causative acid can also be added to the stationary phase instead of the sample solution. We are expanding the application to preparative-scale separations.

REFERENCES

1 Y. Ito, in N. B. Mandava and Y. Ito (Editors), *Countercurrent Chromatography: Theory and Practice*, Marcel Dekker, New York, 1988, Ch. 3, pp. 79–442.
2 W. D. Conway, *Countercurrent Chromatography: Apparatus, Theory and Applications*; VCH, New York, 1990.
3 L. C. Craig and D. Craig, in A. Weissberger (Editor), *Techniques of Organic Chemistry*, Vol. 3, Interscience, New York, 1956, pp. 149–331.
4 H. J. Cahnmann, E. Gonçalves, Y. Ito, H. M. Fales and E. A. Sokoloski, *J. Chromatogr.*, 538 (1991) 165.
5 L. R. Snyder, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, pp. 208–224.
6 T. Kuster and A. Niederweisen, in E. Heftmann (Editor), *Chromatography —Fundamentals and Applications of Chromatographic and Electrophoretic Methods, Part B; Applications*, Elsevier, Amsterdam, New York, 1983, pp. 1–52.
7 C. Galeffi, M. A. Ciasca-Rendina, E. Miranda Delle Monache, A. Villar del Fresno and G. B. Marini-Bettòlo, *J. Chromatogr.*, 45 (1969) 407.
8 G. B. Marini-Bettòlo and C. Galeffi, in F. Bruner (Editor), *The Science of Chromatography*, Elsevier, Amsterdam, New York, 1985, pp. 283–303.
9 N. Catsimpoolas, in Z. Deyl (Editor), *Electrophoresis —A Survey of Techniques and Applications, Part A: Techniques*, Elsevier, Amsterdam, New York, 1979, pp. 167–192.