Bromide Ion Measurements in Animal Tissue Using an Indicator with Thin-Layer Chromatography

A TLC procedure is reported for the analysis of bromide ion in 0.5-g tissue samples in the presence of chloride ion. This procedure involves addition of the indicator bromcresol purple to the solvent system which produces peaks on the chromatoplate. The peak areas can be used to determine bromide ion concentrations, and the sensitivity is improved 50-fold over direct spot spraying. In addition, the results indicate that R_f values of bromide and chloride ions vary with concentration.

The determination of bromide ion at levels below 50 ppm in animal and plant tissue is a formidable task and is especially difficult if one is analyzing 0.5–2-g samples. The most commonly used techniques employ X-ray fluorescence and/or neutron activation analysis. Yet even through the use of these instrumental methods it is not possible to determine levels below 40 ppm. In addition, most laboratories are not equipped with the facilities to conduct the procedures. In this paper we present a method which is sensitive and which utilizes equipment readily accessible to all laboratories. We also describe a phenomenon in thin-layer chromatography (TLC) which to our knowledge has not yet been reported.

Bromide ion is present in plant and animal tissue in levels from 1 to 100 ppm (Hunter, 1955; Lynn et al., 1963). It may be present at higher levels in fumigated foodstuffs (Beckman et al., 1967; Getzendaner et al., 1968), and animals and humans may increase their bromide ion levels after consuming such foodstuffs (Lyn et al., 1963; Getzendaner, 1965). Elevated bromide levels occur in persons exposed to alkyl bromides in the workplace (Hine, 1969) and in animals during alkyl bromide exposure experiments (Lee et al., 1975).

Getzendaner (1975) reviewed the techniques that have been applied to plant tissue, but they are not generally applicable to small animal tissue sample analysis due to poor sensitivity, due to the requirement for large tissue samples, and because of high interfering levels of chloride. A promising technique mentioned by Getzendaner was the use of a bromide selective electrode. This technique is excellent for measuring levels in water samples with low chloride content, but it is not very useful for the analysis of biological tissue without the initial removal of chloride ion. In our efforts to remove the chloride ion using TLC, we found that the bromide levels could themselves be measured directly on the TLC plate.

EXPERIMENTAL SECTION

Reagents. The acetone (Baker) was pesticide grade, while the 1-butanol (Mallinckrodt) and the ammonium hydroxide (Baker) were reagent grade. The water was first distilled and then passed through a Crystalab deionizing filter (FC-10). The halides were all obtained as sodium salts (Mallinckrodt) and were AR grade. The bromcresol purple (Allied) was indicator grade. The radioisotope ^{36}Cl, obtained from the Chemical and Radioisotope Division of ICN, was prepared as the NaCl salt in water. The radiopurity was >99%, the specific activity was 12.7 mCu/g of Cl^-, and the chloride concentration was 0.031 g of Cl/mL of H_2O.

Thin-Layer Chromatography. The chromatoplates were analytical grade with silica gel 60 resin (0.25-mm gel thickness, 20 × 20 cm, Merck) and were scored into 10 equal sections. The halides were separated with the following solvent system (Seiler and Kaffenberger, 1961): 65% acetone, 20% 1-butanol, 10% concentrated ammonium hydroxide, and 5% distilled water. Bromcresol purple (0.02 mL/100 mL of solvent from a 0.1% solution of bromcresol purple in ethanol) was added to the solvent and used as a detector. Prior to use, the TLC plates were run through a cleanup procedure in the solvent system without the indicator.

Sample Analysis. Mouse blood samples were obtained from 27–30-g male Swiss-Webster mice by cardiac puncture using a syringe rinsed with a solution of 1000 units of sodium heparin/mL of saline. Plasma was obtained by centrifugation. The blood samples (0.5 mL of plasma or whole blood) were fortified with 0.5 mL of a bromide ion standard or water. The samples were cooled for 20 min on ice, 2.5 mL of acetone was added to precipitate protein and then the samples were centrifuged (12000g, 10 min, 4 °C). The supernatant was collected and the sample was evaporated to approximately 1 mL, and the acetone precipitation procedure was repeated. The samples were evaporated to dryness and 0.1 mL of deionized water was added. A 10-μL sample was spotted on TLC plates and compared with standards. The mouse liver samples were excised, pooled, and fortified with the bromide ion standard or water. They were homogenized by using a Brinkman Polytron. The protein was precipitated with 4 mL of acetone and the samples were then centrifuged (44000g, 10 min, 4 °C). The supernatant was evaporated to 1 mL of volume, 4 mL of acetone was added, and the samples were centrifuged (100000g, 30 min, 4 °C). The supernatant was obtained and evaporated to approximately 0.4 mL.

Radioisotope Procedure. A 10-μL aliquot of each of several chloride ion standards was spotted on an analytical TLC plate and was developed as explained above. The location of radioactivity was determined by using a Berthold β Camera LB 291. The β-emission photograph was taken by using the Polaroid camera attachment with a 1650 voltage setting, an $f = 8$ aperture, and an exposure time of 10 min.

RESULTS AND DISCUSSION

A typical developed plate is shown in Figure 1. Apparently, the bromcresol purple interacts with ions to create a blue line that has a distinct base line and peaks with areas directly proportional to the concentration of halide ion. The nature of the peaks may be due to formation of an ionic charge complex between the halide and the bromcresol purple. The capillary flow of the bromcresol purple is retarded in areas where the halide is present. The halide–bromcresol purple interaction is consistent with the location of the bromide and chloride ions within the peak as discussed below.

Adding an indicator to the solvent system improved the identification and measurement of halides. This procedure is roughly 50 times more sensitive than the traditional

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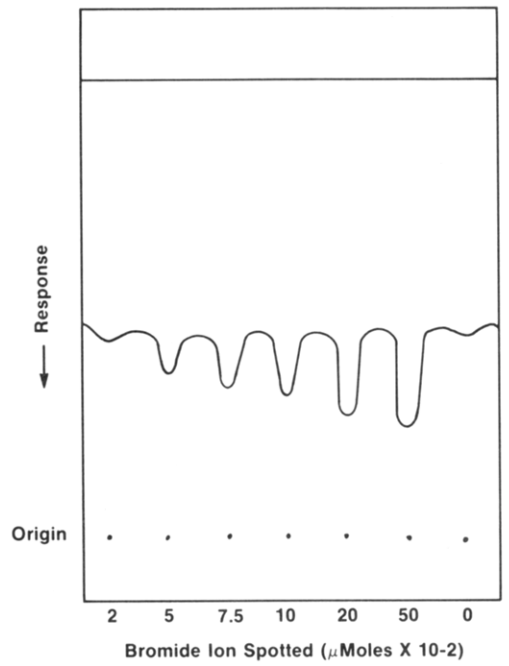

Figure 1. Typical chromatogram of increasing bromide ion concentrations using indicator–TLC.

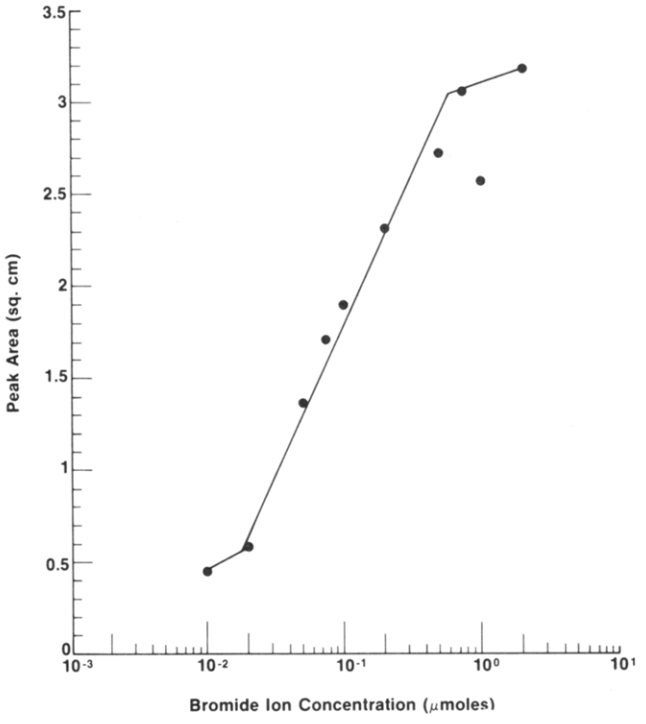

Figure 2. Semilog calibration curve for indicator–TLC determination of bromide ion. 10 μL of a bromide ion standard was spotted in each case. Solvent system: 65% acetone, 20% 1-butanol, 10% ammonium hydroxide, 5% water, and bromcresol purple (0.02 mL/100 mL of a solvent from a 0.1% solution of bromcresol in ethanol).

plate-spraying technique. For quantitation of the areas under the peaks, initially the peaks were traced onto paper, cut out, and weighed. The weight was then plotted against concentration. Presently we are using an x–y plotter to input points from the peaks into a Tektronix 4052 mini computer system. By use of the points, the area under the peaks is calculated in square centimeters. Both methods allow plotting a semilog graph similar to that shown in Figure 2. The results are linear from 0.02 to 0.50 μmol of bromide. At bromide concentrations greater than 0.50 μmol, there is an increase in area, but it is not longer linear. At concentrations below 0.01 μmol, a peak is not longer visible.

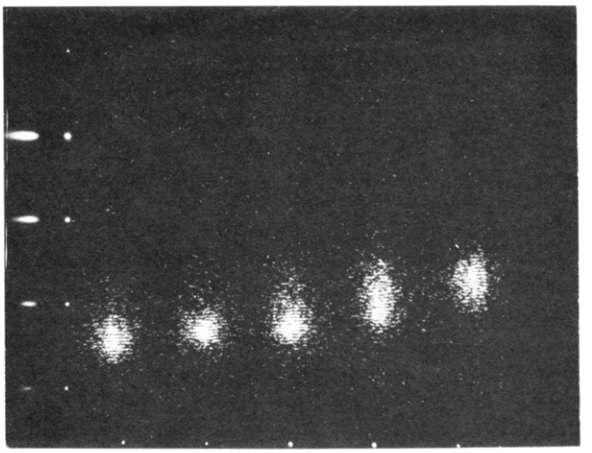

Figure 3. Radiograph of thin-layer chromatogram with 10-μL spots of varying concentrations of chloride ion. Following plate development the radioactivity was visualized by using a Berthold β Camera LB 291 (voltage setting = 1650 V; aperture of f = 8; exposure time = 10 min). From left to right the samples represent 5.0, 1.0, 0.5, 0.1, and 0.01 μmol of chloride ion.

The R_f values of bromide and chloride ions were found to be dependent on concentration. When bromide ion peaks were sprayed with bromcresol purple, the spot corresponded to the tip of the peak. The R_f values decreased from 0.336 when 0.05 μmol was spotted to 0.303 when 1.0 μmol was spotted. The R_f values remained constant at 0.303 when concentrations from 1.0 to 10.0 μmol were used. Concentrations below 0.5 μmol were not visible. The R_f values obtained with 0.01–5.0 μmol of chloride were also inversely proportional to concentration as shown in Figure 3. At concentrations below 0.01 μmol the R_f values do not increase further, while at concentrations greater than 5.0 μmol the R_f values no longer decrease. The location of radiolabeled chloride ion was within the area under the peak, toward the apex. The varying R_f values are not due solely to the presence of bromcresol purple. When radioactive chloride is spotted on TLC plates and developed without bromcresol purple, the R_f values still decrease with increasing concentrations, but differences are greater when bromcresol purple is used.

This TLC procedure eliminated all major interferences. Chloride ion has a much lower R_f value and the chloride ion peaks do not interfere with the bromide ion peaks. When spotted together, the location of each ion is visible. The following ions were determined not to pose any interference problems: fluoride (0.0142 mM), chloride (0.113 M), iodide (5.52×10^{-4} mM), nitrate (0.0001 M), sulfate (0.021 M), phosphate (0.052 M), and bicarbonate (24.0 M).

By use of a 0.5-g sample of mouse blood or mouse plasma, bromide levels were detected at the 40-ppm level. Bromide concentration levels were studied in plasma, whole blood and liver, and recoveries are listed in Table I. The recovery for bromide ion in plasma averaged 88.0%. For the mouse whole blood the average recovery was 78.2%. The recovery for bromide ion in liver averaged 95.2%. Background levels of bromide ion were not detected by the indicator–TLC method or by X-ray fluorescence (in liver tissue). A small peak, distinguishable from a bromide ion peak, is obtained when unfortified tissue samples are spotted by using the indicator–TLC method.

The indicator system's limit of detection is about 2×10^{-2} μmol. The practical limit of detection will vary de-

Table I. Recovery of Bromide Ion from Tissue

tissue	fortification, μmol/g	detected,[a] μmol/g	% recovery
mouse plasma	0	ND[b]	
	0.5	0.430 ± 0.017	86.0
	1.0	0.740 ± 0.075	74.0
	1.5	1.562 ± 0.089	104.1
mouse whole blood	0	ND[b]	
	0.5	0.317 ± 0.033	63.3
	1.0	0.759 ± 0.037	75.9
	1.5	1.431 ± 0.155	95.4
mouse liver	0	ND[c]	
	0.5	0.342 ± 0.102	68.4
	1.0	0.573 ± 0.102	57.3
	3.5	2.835 ± 0.116	81.0
	6.2	5.679 ± 1.090	91.6
	8.2	6.749 ± 0.918	82.3

[a] Mean ± standard error. [b] ND, no bromide ion detected. [c] ND, no bromide ion detected by indicator-TLC or X-ray fluorescence.

pending on the type of sample used, the background level, and the quantity of sample available for analysis. This system is very useful for tissue samples from small laboratory samples, particularly mice.

Aside from evaluating blood samples, this TLC system allows measurement of liver bromide ion concentrations. Measuring ion concentrations in the liver is generally complicated by the large number of interfering ions, lipids, and proteins. The centrifugation procedure removes the larger molecules and the TLC procedure separates the interfering ions. Bromide levels in human serum can be analyzed if the samples are handled in a manner similar to that for the liver samples. High lipid tissues are more difficult to spot and analyze, particularly when concentrated to very small volumes. This problem is minimized with ultracentrifugation of the sample. Some presently unidentified ionic compounds may also increase the background levels of the sample.

As seen in Figure 1, there is a slight background interference when water only is spotted. This background may also be present without spotting a sample and may be due to the variable thickness of the TLC plate. This background was determined and subtracted from our samples for each experiment. Since there is a slight variation for each experiment, the peak areas should be calibrated for each experiment with a new standard curve.

This TLC procedure has been tested with chloride and bromide ions, and it is our feeling that it may be useful for other halides. The type of procedure may be applicable to other systems where different compounds are separated and where an indicator is used for detection. Modification of an existing TLC procedure may increase the sensitivity of the system and allow a more precise means of quantification.

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A Fully Automated High-Performance Liquid Chromatographic Procedure for Isolation and Purification of Amadori Compounds

A semipreparative reversed-phase high-performance liquid chromatographic (HPLC) method was developed for the separation and purification of Amadori compounds from crude extracts of Maillard reactions. The Amadori compounds alanine–fructose (Ala–Fru), leucine–fructose (Leu–Fru), hydroxyproline–fructose (Hyp–Fru), and tryptophan–fructose (Trp–Fru) were isocratically separated on a NH_2-bonded silica gel column by using methanol–water (80:20) as the mobile phase. The collected fractions containing the Amadori compounds were concentrated and chromatographed a second time under the same conditions for further purification. Pure products were obtained by crystallization from anhydrous methanol of the fractions obtained after evaporation. The purities of the Amadori compounds were tested by thin-layer chromatography (TLC) and HPLC; their structures were confirmed by infrared spectrometry, ^{13}C NMR, and mass spectrometry.

Amadori compounds (1-amino-1-deoxy-2-ketoses) occurring in the early stages of the nonenzymatic browning Maillard reaction, have been isolated from several biological materials (Abrams et al., 1955; Anet and Reynolds, 1957; Heyns and Paulsen, 1959; van den Ouweland et al., 1979).

Some of their properties explain the growing interest in these compounds: they are considered as potential "natural antioxidants" (Hodge, 1955; Eichner, 1975) and precursors of the aroma and flavor of processed foods. An important development has been made in the chemistry of aromas in Maillard model systems (Tressel, 1979; Po-

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