Improved version of the Kean partition assay for cerebroside sulfate

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Summary In the Kean method for the colorimetric determination of sulfatide (1968. *J. Lipid Res.* **9:** 319–327), the lipid is partitioned together with a blue cationic compound between two phases formed from chloroform, methanol, and water. The blue cation enters the chloroform-rich phase only as an ion pair with the lipid. This method has been improved by the use of a new mixture of solvents in which the desired layer floats above the excess dye. The lower volatility of the new solvent system improves the reproducibility of the technique.—**Radin, N. S.** Improved version of the Kean partition assay for cerebroside sulfate. *J. Lipid Res.* 1984. **25:** 651–652.

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The technique of chromogenic ion-pairing by liquid/liquid partitioning was adapted by Kean (1) for analysis of brain lipids for their content of galactocerebroside sulfate (sulfatide). In this procedure, azure A, a blue hydrophilic diamine, is partitioned between chloroform, methanol, and 25 mM aqueous sulfuric acid. Only a little color appears in the lower, chloroform-rich phase when sulfatide is absent. When sulfatide is present, the blue amine is pulled into the lower phase as a salt, although most of the azure A remains in the upper phase. Transfer of the lower phase must be done without any contamination by the very dark upper phase if high values are to be avoided. As the upper phase is removed, evaporation of the chloroform tends to cool it and precipitate out the water normally solubilized by the methanol which is also present in the lower layer. There is also some danger of differential evaporation of the nonpolar phase, so that some samples might yield erroneously high absorbance values. These problems led us to develop a variant of the system in which higher-boiling solvents are used, with a nonpolar phase which floats and is more easily transferred to the spectrophotometer.

Isooctane (2,2,4-trimethylpentane, J. T. Baker, spectrophotometric reagent, bp 99°C) was initially chosen for the upper phase. However, this hydrocarbon was not polar enough to dissolve the sulfatide-amine salt, so chloroform was added. Because of its high density, the amount added was limited and the mixture proved to be insufficiently polar to produce a useful partition constant for the salt. The inclusion of isoamyl alcohol (3-methylbutanol, J. T. Baker reagent, bp 131°C) and 2-propanol (bp 82°C) yielded more intense blue colors in the upper phase, indicating that a more complete transfer of the salt had occurred.

Kean reported that methylene blue, which has the same structure as azure A except for two additional N-methyl groups, yielded the same absorbance with his partition system. He also noted that batches of azure A from different suppliers differed somewhat in color yield, evidently due to the typical problems of dye manufacture. While we found the latter dye to be satisfactory, we thought that the more common amine might be more likely to be of consistently good quality. We used methylene blue from Fisher Scientific.

Samples containing zero to 60 μg of sulfatide, prepared as the Na salt (2), are evaporated from solution with a stream of nitrogen in a borosilicate disposable test tube, 13 × 100 mm. To the residue are added 2 ml of upper phase (isooctane–chloroform–isoamyl alcohol–isopropanol 3:1:1:1, v/v) and 1 ml of lower phase (0.2 mg methylene blue in 1.25 mM sulfuric acid), using repeating dispensers. The tubes are capped with foil-covered rubber stoppers, vortexed at least 0.5 min, and centrifuged briefly to separate the two phases. Care should be exerted to avoid getting liquid onto the stopper. Most of the upper phase (still 2 ml) is transferred to a cuvette having a 1-cm path length and the absorbance is determined at 668 nm. The blank cuvette is filled with water rather than pure upper phase, to avoid changes due to evaporation.

Linearity of the method was tested with duplicate sulfatide samples weighing 0, 10, 20, 40, and 60 μg. A straight line was obtained with a slope of 0.0332 absorbance units per microgram and a correlation coefficient of 0.99972. The absorbance of the blanks was about 0.019.

In the case of natural samples, which include acidic phospholipids, one obtains high values for sulfatide, as pointed out by Kean in his very thorough study (1). Alkali-catalyzed methanolysis seems like the simplest method for eliminating this kind of interference. There remains the possibility, however, that the methyl esters thus produced and the alkali-stable lipids might interfere with the dye-partitioning phenomenon. This was tested with brain lipids by analyzing aliquots of a hexane–isopropanol extract (3, 4) of calf brain for sulfatide content. Four aliquots, each corresponding to 20 mg of brain, were evaporated to dryness, treated with NaOH in chloroform–methanol for 30 min, neutralized with aqueous HOAc, and the lower layer was washed with methanol–water and evaporated to dryness (5). Two of the four aliquots were added to 60 μg of sulfatide prior to the initial evaporation. The residues were dissolved in 8 ml of chloroform–methanol

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and duplicate 2-ml portions were evaporated to dryness in 13 × 100 mm test tubes and analyzed by our method, together with a set of sulfatide standards. Volumetric pipets were used for all portion measurements (except for the dispensing of the two partitioning solvents) to maintain good precision.

The sulfatide content in the brain samples alone was found to be 15.08 μg ± 2.0% CV (individual values = 14.68, 15.00, 15.31, and 15.31 μg). Correcting for the 2-ml aliquots from 8 ml of sample, this corresponds to 60.3 μg and a (typical) concentration of 3.02 mg/g brain. The mixture of brain extract with sulfatide should have yielded a final value of 60 + 60.3 = 120.3 μg, but the observed value was 118.6 μg ± 1.4% CV (individual values in the analyzed samples = 29.06, 29.72, 29.79, and 30.02 μg). Thus the observed recovery of added sulfatide was 58.3 μg, or 97% of the amount added. This shows that little interference is produced by the non-sulfatide lipids in the brain sample, and that there is little loss of sulfatide during the alkaline methanolysis procedure.

While Kean indicated that the inclusion of dilute sulfuric acid was really not necessary, we found that methylene blue (but not azure A) produced a visible emulsion at the higher sulfatide levels unless a trace of acid was included.

The good reproducibility of the assay (indicated by the individual values shown above) suggests that useful measurements can be made with samples containing as little as 1 μg of sulfatide, equivalent to about 0.25 mg of adult rat brain. A limiting factor is the precision of the two dispensers of the two liquid phases. The use of a flow sampler in a spectrophotometer could double or triple the sensitivity by allowing the use of smaller solvent volumes. Additional sensitivity could be attained if a fluorescent amine could be found with properties like those of methylene blue.

A recently described modification of the Kean method, in which the volume of the chloroform layer was reduced to about 0.7 ml, yielded a specific absorbance of 0.110 absorbance units per microgram (6). A useful feature of this new method is that the partition technique could be applied to more polar sulfolipids by acetylating them first.

The partition solvents described in the present Note may prove useful for other chromogenic partition assays.

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