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Letter to the Editor

Use and regeneration of Amicon ultrafiltration cones for deproteination of microsomal solutions before chromatographic analysis

Sir,

Recently, we have worked on the development of an assay for measuring quantitatively the formation of monohaloacetaldehyde and monohalooxirane from the microsomal metabolism of various carcinogenic haloaliphatics, such as vinyl chloride, 1,2-dibromoethane, 1,2-dichloroethane, and the clinically used N-mustards [1]. The assay is based on the fact that monohaloacetaldehyde [2, 3] and monohalooxirane [4] will react with adenine compounds to form highly fluorescent 1,N^6-ethenoadenines. Consequently, in our assay, typical microsomal metabolism is allowed to take place in the presence of an adenine, after which the solution is acidified and heated to maximise the efficiency of the fluorochromogenic reaction. Cyclic AMP has been found to be a suitable adenine in terms of its solubility and its stability in the post-metabolic steps. The product of the trapping, 1,N^6-etheno-cAMP, is then measured using high-performance liquid chromatography and a fluorescence detector.

A critical aspect in the development of this assay was the choice of the method for deproteinating the acidified microsomal solutions. It was anticipated that the typical experiment would have many samples that would need to be deproteinated before chromatographic analysis. Hence, it was desirable to have a quick method for deproteination. Hartwick et al. [5] have reviewed the various strategies for deproteinating biological samples and have compared several of the more popular methods. Ultrafiltration using Amicon Centriflo membrane cones CF25 was found to be easy, efficient and rapid. Also, the ultrafiltration method did not involve any dilution of the sample with solutions of salts or solvents, which also could be sources of interfering peaks during chromatography. Consequently, ultrafiltration was tried as the method for deproteination in our assay and has proven to be quite useful, provided several properties of the cones are appreciated. Given the growing use of ultrafiltration cones in the processing of biological materials for chromatographic analysis [5—7] as well as their potential use in microsomal studies such as our own, we thought that our experiences in using and regenerating these devices would be of interest to your readers.

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First, the cones should be pre-dried by centrifugation immediately before use so as to avoid a slight dilution of the sample because of their residual wetness (ca. 0.1 ml). We pre-dry cones by standing them inverted on paper towels for ca. 5 min before centrifuging them in their supports for 10 min at 750 *g* (Sorval RC-5 with a type SS-34 head).

Second, microsomal solutions (1 ml) with protein concentrations of $\leqslant 2$ mg/ml have been deproteinated at 4°C by centrifuging for ca. 35 min at 750 *g*. The resulting filtrate is clear and suitable for immediate injection onto a column. If suspected, the presence of protein can be assessed qualitatively by the development of a blue colour when 0.1 ml of the filtrate is combined with 0.1 ml of undiluted Bio-Rad protein assay dye (Coomassie Brilliant Blue G-250) [8].

Third, the cost of the cones and the number of samples that could be expected in the course of an ongoing study of microsomal metabolism dictate that the cones be reusable. However, the methods recommended in the product literature for regenerating the cones, soaking in 0.1% sodium hydroxide or 3—5% sodium chloride, are designed to remove soluble proteins, but neither method is effective with microsomal protein solutions. What occludes the pores of a cone after ultrafiltration of a microsomal protein solution is not simply protein but rather the microsomes themselves, which are microspheres of intracellular membrane embedded with proteins. The lipid nature of the microsomes suggested that a lipase solution could be used to digest the occluded material. We have regenerated the cones with the following enzymatic procedure.

Cones in their supports were placed atop 50-ml polycarbonate tubes that had been filled with 100 m*M* potassium phosphate (pH 7.5). The cones were then filled with a solution of 1% (w/v) lipase (Sigma product number L3126) in the same buffer. Parafilm was stretched over the cones to prevent evaporation, and the cones in their polycarbonate tubes were incubated overnight (15—24 h) at 37°C. By the end of the incubation, the cones had developed an offensive odour due to some microbial growth. The contents of the cones and the polycarbonate tubes were discarded. The cones were rinsed in running tap water for 5 min, placed in 1-l beakers full of 1 m*M* sodium hydroxide in 50% aqueous ethanol, and agitated for a 30 min in a sonic bath (Heat Systems Ultrasonics; 60 Hz). The contents of the beakers were discarded, the cones were rinsed again in running tap water for 5 min, and the 30-min sonication in freshly made alkaline, aqueous ethanol was repeated. Finally, the cones were rinsed in running tap water for 5 min and stored in distilled water in a refrigerator at 4°C.

The enzymatic method described above has proved so successful that some of the cones have been used for over one year, during which time they were regenerated over twenty times. Only one cone in the forty cones that were actively used during our research was not regenerated. This failure occurred only after thirteen previous regenerations. This cone was easily recognized by a decrease in both the volume of its filtrate and in its ability to transmit an ethenoadenine standard. To date, none of the forty actively used cones has been observed to develop a leak, which would manifest itself by an increase in the volume of the filtrate as well as the presence of protein in the filtrate.

Fourth, using $1,N^6$-etheno-cAMP, the product of the trapping reaction of our

TABLE I

THE LOSS IN TRANSMISSION OF 1,N^6-ETHENO-CYCLIC AMP BY ULTRAFILTRATION CONES THAT HAVE BEEN REGENERATED ENZYMATICALLY

A solution containing microsomes (1 mg/ml) and 1,N^6-etheno-cAMP was acidified to pH 4.0 and deproteinated by ultrafiltration using cones with different histories of use as indicated. Transmission of 1,N^6-etheno-cAMP into the filtrate was determined chromatographically [1]. The average transmission for unused cones is set arbitrarily to 1.00 and all values are reported relative to it as the average for four cones (± the standard deviation).

Number of previous regenerations	Relative transmission
0	1.00 ± 0.01
1	0 98 ± 0 02
10	0.91 ± 0.02
⩾20	0 93 ± 0.02

assay, we have noticed that there is a slight difference in terms of the transmission between new, unused cones and those that have been regenerated (Table I). This difference between cones, depending on their number of regenerations, contributes to the variance of our assay but the magnitude is such that it is inconsequential when cones are randomly assigned for deproteinating microsomal solutions. Therefore, in order to randomize cones and otherwise keep a history of their use, we have numbered our cones by a hole-punch scheme on their rims.

Finally, it should be noted that the cones (unused as well as regenerated) do not transmit all of an ethenoadenine standard into their filtrate. A similar situation was observed by Hartwick et al. [5] for the recovery of the purine theophylline from human serum. The loss in transmission in our assay has at least two components: adsorption on the cones and adsorption on the microsomes. The latter may include a pH-dependent binding to proteins [5].

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