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Note

Determination of saccharin in diet and biological materials

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Sodium saccharin has been used as a non-nutritive sweetening agent for over 100 years. When fed at high levels in the diet, it has been shown to be a promoter of bladder tumors in Fischer 344 rats in a two-stage model of carcinogenesis. In addition, sodium saccharin administered to rats in the diet over two generations causes a significant increase in bladder tumors[1]. There is, however, no evidence that sodium saccharin contributes to the incidence of human neoplasms[2]. Further research into the mechanisms by which this agent causes bladder cancer in experimental animals is required.

Feeding studies in our laboratory involving large numbers of experimental animals necessitate fast, quantitative analysis of saccharin in biological materials and in the diet (to ensure correct concentration and uniform distribution). Various methods have been published[3–8], but they are either tedious, requiring large amounts of organic solvents for extraction, or lack sensitivity.

This paper describes development of a new method for extraction of saccharin from various matrices prior to analysis by high-performance liquid chromatography (HPLC) and quantitation by fluorescence detection.

EXPERIMENTAL

The following specimens, obtained from rats fed a diet containing sodium saccharin, were analyzed for saccharin content: serum, feces, milk, and urine as well as the diet itself.

Fischer 344 rats (4 weeks of age, 40–50 g body weight) were obtained from Charles River Labs. (Kingston, NY, U.S.A.) and maintained in quarantine for one week prior to being fed Prolab 3200 diet (Agway, St. Mary's, OH, U.S.A.) containing sodium saccharin (PMC Specialties Group, Cincinnati, OH, U.S.A.) at a level of 5% by weight. The diet was mixed and pelleted by Dyets (Bethlehem, PA, U.S.A.). Urine and feces were collected separately, using a stainless-steel metabolic cage (Model

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LC176, Lab Products, Maywood, NJ, U.S.A.). Toluene (2 ml) was added to the urine collection tube as a bacteriostatic agent prior to initiation of the metabolic study. Feces were air dried (50°C, 17 h) prior to extraction. To obtain milk, pregnant rats were purchased from Charles River and maintained through 13 days post-partum. Milk was collected manually from the nipples of lactating female rats into a capillary tube prior to sacrifice. The milk was transferred to a 1.5-ml microcentrifuge tube and stored at −70°C until required. Blood was collected by aortic puncture from anesthetized animals at termination of the study. The blood was transferred to a tube, and the serum collected by centrifugation (1000 *g*, 10 min in a Sorvall T6000 centrifuge, DuPont, Wilmington, DE, U.S.A.) and stored in 10 ml polypropylene tubes at −70°C until required.

A sample (approximately 350 mg) of diet or dried feces was weighed, prehydrated with 0.35 ml of 100 m*M* sodium hydroxide and extracted three times with 2.5 ml aliquots followed by a 2.0-ml aliquot of 100 m*M* sodium hydroxide. The sample was centrifuged (500 *g*, 10 min) between each extraction; the supernatants were combined in a 10-ml volumetric flask and diluted to volume with triple distilled water. An aliquot of urine (1 ml) or serum (0.5 ml) was placed into a 10- or 5-ml volumetric flask, respectively, 100 m*M* sodium hydroxide (8.5 ml or 4.25 ml, respectively) was added and diluted to volume with water. A volume of 1 ml of a 122-m*M* stock solution of sodium saccharin was treated in the same manner as the urine for use as a standard. Equivalent amounts of sample matrix obtained from control animals were spiked with a known amount of saccharin, 25 mg, for determination of extraction efficiency.

An aliquot (0.5 ml) of diet or feces extract, diluted urine, standard, or undiluted rat milk (0.2 ml) was applied to a 3-ml disposable quaternary amine column (J. T. Baker, Phillipsburg, NJ, U.S.A.) that had been conditioned by sequential washes (3 ml) with methanol, ammonium hydroxide (sp.gr. 0.9), water, and 200 m*M* hydrochloric acid[9]. After sample application, the column was washed with two 1-ml aliquots of methanol, followed by a 2-ml aliquot of water with 3 μl of 2-octanol added on top of the water in each column as an anti-foaming agent. The saccharin was eluted into a volumetric flask (5 ml) with two 2-ml aliquots of 200 m*M* dibasic potassium hydrogenphosphate buffer (pH 8.8). The pH of the eluate was adjusted to 2.0 with phosphoric acid (80 μl) and the sample diluted to volume with HPLC-grade water.

HPLC was performed on a Waters system (Milford, MA, U.S.A.) consisting of Model 510 pumps, a WISP 712 automated sample injector or U6K manual injector, temperature control unit, and oven. A Waters Novapak 4 μ end-capped C_{18} column (15 cm × 0.39 cm I.D.) was eluted isocratically at 30°C with acetonitrile–10 m*M* monobasic potassium hydrogenphosphate (16:84), pH 2.2, at a flow-rate of 1 ml/min. The mobile phase was made using only HPLC-grade solvents and was filtered and degassed prior to use.

Saccharin was detected by using either a Waters 481 UV spectrophotometer (254 nm) or a Perkin-Elmer, Model LS5B, spectrofluorometer (ex, 232 nm; em, 432 nm). The UV wavelength was obtained from published data[4], and the fluorescence excitation and emission maxima were determined using a prescan facility on the LS5B. These detectors were interfaced with a Hewlett-Packard 3390A integrator to provide on-line reporting of data.

Electrochemical detection was also considered, but analysis of saccharin by cyclic voltammetry without derivatization showed no oxidation potential. Cyclic voltammetry analyses were run on a CV-27 voltammograph (Bioanalytical Systems, Lafayette, IN, U.S.A.), equipped with a standard three-electrode cell with glassy carbon working electrode, platinum wire auxiliary electrode, and silver/silver chloride reference electrode. Voltammograms were recorded on an XY recorder, Model 100 (Houston Instruments, Houston, TX, U.S.A.). Acetonitrile (Aldrich, Milwaukee, WI, U.S.A.) was distilled from calcium hydride just prior to use. Commercially available (Aldrich) tetrabutylammonium hexafluorophosphate (tBu_4NPF_6) was used without further purification.

A standard procedure was followed for analysis by voltammetry: a 1–2 m*M* solution of saccharin (as the free acid or sodium salt) in 0.1 *M* tBu_4NPF_6 in acetonitrile was degassed by bubbling argon into it for 10 min with stirring. Bubbling was stopped and a constant positive pressure of argon was maintained over the solution so as to prevent contamination with oxygen. While monitoring the output current, the initial potential, 0 V, was applied to the cell; when the resulting charging current had decreased to zero, the scan was started at a sweep rate of 200 mV/s. The analysis potential was between -1.00 and $+3.00$, and the temperature was maintained at 22 ± 2°C.

RESULTS

The binding capacity of the quaternary amine solid phase extraction (SPE) column was determined for each matrix by placing varying amounts (250–2000 μl) of extract on the columns (see Fig. 1). The diet extract had the lowest binding capacity of the samples tried. The standard curve for each of the samples was linear up to about 1 ml of extract, so 0.5 ml of extract was used as the amount applied to the SPE column for routine analysis. The assay was validated using diet, feces, and urine since they were readily available for use.

Intra- and inter-run reproducibility, as well as recovery, were determined for

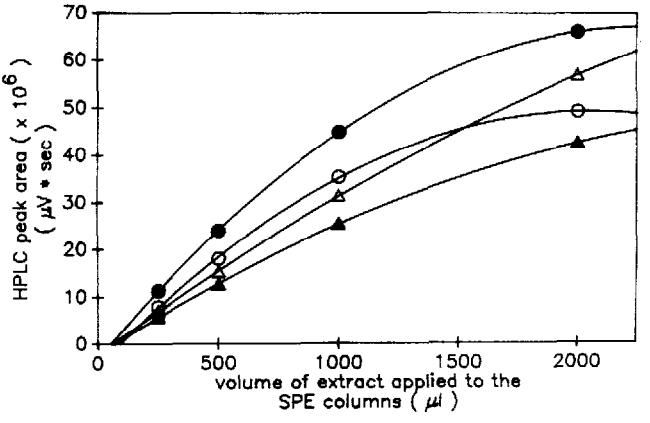

Fig. 1. Binding curves of the SPE columns. (●) Urine extract; (△) feces extract; (○) diet extract; (▲) standard (0.25 μg/μl).

TABLE I

STATISTICAL EVALUATION OF SACCHARIN ASSAY

X = mean; S.D. = standard deviation; C.V. = coefficient of variation; ND = not determined; n = 10 for both intra- and inter-run variation.

		Intra-run variation	*Inter-run variation*	*Recovery*
Diets	X	7.51%	7.2%	89.8%
	S.D.	0.26%	0.3%	2.5%
	C.V.	3.5%	4.6%	2.7%
Urine	X	20.3 mg	21.0 mg	93.9%
	S.D.	0.4 mg	0.5 mg	4.4%
	C.V.	2.0%	2.2%	4.7%
Feces	X	24.6 mg	ND	98.6%
	S.D.	0.4 mg	ND	1.6%
	C.V.	1.6 mg	ND	1.6%

each of the matrices. Diet containing 7.5% sodium saccharin by weight was used for statistical analysis. For intra-run reproducibility, ten samples of pooled urine, crushed feces, or crushed diet were extracted, and the saccharin content was determined from the average of four injections per sample (see Table I). Inter-run reproducibility was evaluated in the same way as intra-run determination except that each extraction and analysis were performed on separate days over a 10-day period (see Table I). Recovery for each matrix was determined by dividing the spiked control sample's peak area by the standard peak area and multiplying the result by 100. The saccharin content in mg was determined from the equation:

$$(A_x/A_s) \cdot \text{standard concentration} \cdot D \cdot \text{MW} = \text{mg of saccharin}$$

where A_x = the sample peak area on HPLC, A_s = the standard peak area on HPLC, D = the dilution incurred during the extraction process (100 ml), and MW is the molecular weight of the saccharin salt used (205.173 for sodium saccharin, 183.184 for acid saccharin). The standard concentration of saccharin used was 1.22 mM.

Extraction efficiency for the procedure was determined for each sample matrix and found to be in the range of 87–99% for diets and most other matrices, and between 75–90% for milk samples.

The standard curve for HPLC analysis of saccharin was linear with UV and fluorescence detection. The lower limit was 245 pmol (see Fig. 2a) with UV detection and 2.45 pmol (see Fig. 2b) with fluorescence detection.

DISCUSSION

A method for the rapid extraction and sensitive quantitation of saccharin in animal diets and biological materials has been devised. Published procedures[3–7] rely

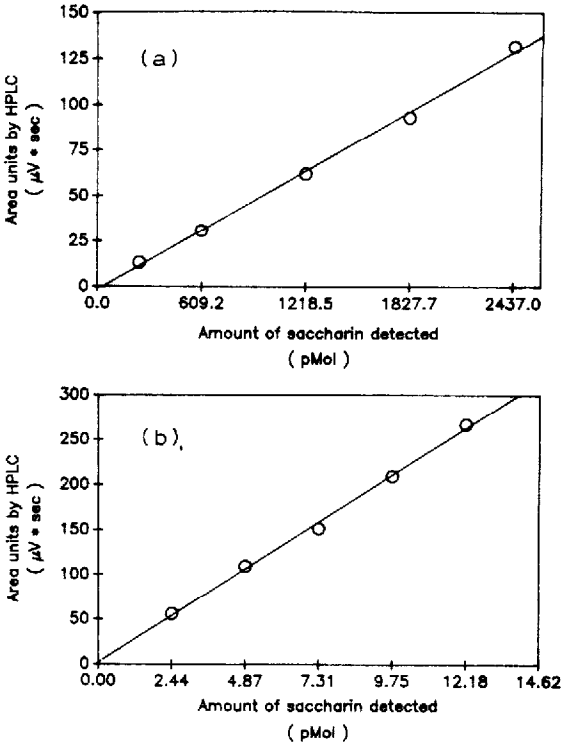

Fig. 2. Lower limits of saccharin detection by (a) UV detection at 254 nm, and (b) fluorescence detection; excitation, 232 nm; emission, 432 nm.

upon partition of the neutrally-charged saccharin molecule from acidic solution into an organic solvent, such as diethyl ether, prior to column chromatography. Since the pK_a of saccharin is 2.2 (ref. 10), it exists as an anion in neutral or basic aqueous solution and can be isolated from biological fluids and alkaline extracts of solids, such as diet or feces, by SPE on a strong anion-exchange column. Compared to organic solvent extraction, this solid-phase procedure provides substantial savings in use of organic solvents and allows a greater number of samples to be analyzed per unit time. Potentially, this procedure can be used for the concentration of dilute solutions of saccharin prior to quantitation and would be limited only by the binding of other components in the sample matrix to the SPE column.

The lower recovery of saccharin from milk is probably due to competitive binding of proteins to the SPE column. Since the saccharin content of milk is high (1 mg/ml), this problem can be resolved by dilution of the milk prior to extraction and the use of fluorescence rather than UV detection.

The use of a 4 μ end-capped C_{18} reversed-phase column reduces HPLC analysis time and provides a sharper peak by reducing adsorption compared to the non-end-capped C_{18} column used in a previously reported method[4]. Run times are typically 4–5 min with the saccharin peak eluting between 1.75 and 2.10 min (see Fig. 3). The sensitivity of the assay can be increased 100-fold by the use of fluorescence

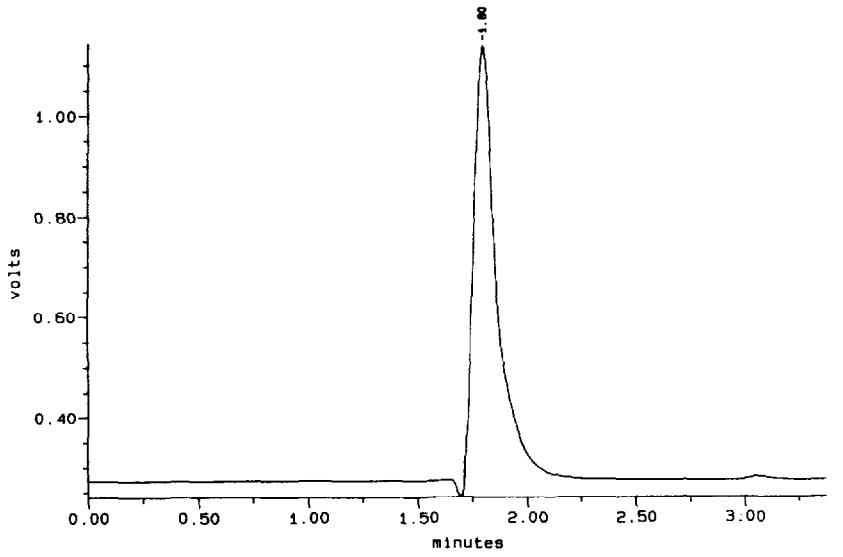

Fig. 3. Representative HPLC chromatogram of saccharin extracted from diet, detection by fluorescence (excitation, 232 nm; emission, 432 nm).

rather than UV detection. This chromatographic procedure is ideally suited for automation, because of the short run times and the large number of samples that can be extracted in a single day.

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