Journal of Chromatography, 238 (1982) 500–505
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,618

Note

Assay of saccharin and sodium saccharin in animal feed*

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(Received December 7th, 1981)

Continued research about possible carcinogenic activity of saccharin necessitated the development of a reliable and accurate assay method for this compound in animal feed diet mixtures used in these studies. It is known that in long-term studies of this nature drug administration to laboratory animals is usually done by incorporation into feed because of the inconvenience of individual manual dosing and the time involved in preparing individual dosages. Analytical methodology is required to determine that appropriate dosages are administered and that the drug is uniformly distributed and stable in the feed mix under the storage conditions used.

Various gas–liquid chromatographic methods were published recently for the assay of saccarin[1–3]. These methods, however, required prior derivatization of the compound. Within the last few years many high-performance liquid chromatography (HPLC) methods have been reported for the quantitation of saccharin. These HPLC methods dealt mostly with biological fluids, beverages and similar samples[4–8] which may not be applicable for animal feed.

Very recently, Holder and Bowman[9] developed an HPLC method for the assay of saccharin in animal feed. This method employed a 2-step extraction of saccharin from the feed sample; first into an aqueous sodium carbonate solution, followed by extraction into ether after acidification. The HPLC step utilized a paired-ion mobile phase and the recovery data were relatively low: 65.0–87.3% for 10.0- and 75,000-ppm samples, respectively. In addition, for samples less than 1000 ppm the feed extract was subjected to a silica gel column cleanup. This paper reports a reversed-phase HPLC method using glacial acetic acid–aqueous methanol mobile phase, after a one-step extraction and a simple cleanup procedure, with sulfathiazole as internal standard. The method resulted in higher percent recovery data.

EXPERIMENTAL

Apparatus

The following were used: a Waters Assoc. Model ALC/GPC 244 liquid chromatograph with a U6K injector, M 6000 pump and M 400 fixed-wavelength UV detector (254 nm); a Houston Instruments (Austin, TX, U.S.A.). Omniscribe Series

* To be presented at the *Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Las Vegas, NV, Meeting*, April 1982.

0021-9673/82/0000–0000/$02.75

A-5000 strip-chart recorder; a glass chromatography tube, 350 × 15 mm I.D., with a PTFE stopcock: a mixer (Vortex-Genie, Scientific Industries, Springfield, MA, U.S.A.); a centrifuge; a rotary evaporator; and 50-ml centrifuge tubes fitted with polytef-lined screw caps.

Reagent and materials

The following were used: saccharin (Eastman Kodak, Rochester, NY, U.S.A.); sodium saccharin dihydrate (Fisher Scientific, Fair Lawn, NJ, U.S.A.); sulfathiazole (Sigma, St. Louis, MO, U.S.A.); diatomaceous earth (Celite 545 AW; Supelco, Bellefonte, PA, U.S.A.); diatomaceous earth (Celite 545 AW; Supelco, Bellefonte, PA, U.S.A.); animal feed (Purina Laboratory Chow, Ralston Purina, St. Louis, MO, U.S.A.); acetic acid–methanol–water (2.5:100:500); acetic acid–ethyl acetate–chloroform (1:7.5:25); and methanol–water (1:1). All other chemicals used were analytical grade.

HPLC conditions

A 25 cm × 4.5 mm I.D., 10-μm Partisil ODS-II ITP (Jones Chromatography, Columbus, OH, U.S.A.) was used at ambient temperature. An isocratic mobile phase system of acetic acid–methanol–water (2.5:100:500) was delivered at the rate of 1.2 ml/min. This mobile phase was filtered through a 5-μm membrane filter (Miltex Type LS, Millipore, Bedford, MA, U.S.A.) and degassed before used. The detector was attenuated to 0.02 and 0.1 a.u.f.s. for the 200- and 2000-ppm samples, respectively.

Internal standard solution

A solution of 0.02 or 0.1 mg of sulfathiazole/ml of methanol–water (1:1) was prepared for the 200- and 2000-ppm samples, respectively.

Standard solution preparation

About 10 mg of saccharin was weighed accurately into a 10-ml volumetric flask and dissolved in diethyl ether. The solution was diluted to volume with ether. A 1-ml volume of this stock solution (for use with the 2000-ppm sample) or 1.0 ml of a 1:10 diluted stock solution (for use with the 200-ppm sample) was pipetted into a 100-ml boiling flask and carefully evaporated to dryness on the rotary evaporator. Ether traces were removed under a nitrogen stream. The residue was subjected to the same cleanup and chromatographic procedures described for the residue from the sample.

Diatomaceous earth column preparation

A small glass wool plug was placed at the base of the glass chromatographic tube. A 2-g amount of diatomaceous earth AW was transferred to the tube and tamped lightly. A 3-g amount of diatomaceous earth AW and 1.5 ml of 0.1 *M* of sodium hydroxide were mixed gently and transferred to the tube. The tube was tapped on a padded bench to settle the packing and them tamped very gently but evenly.

Sample preparation

About 15 ml of 7.5% (w/v) hydrochloric acid was placed in a 50-ml centrifuge tube. About 5 g of a 200- to 2000-ppm animal feed sample was weighed accurately and transferred into the centrifuge tube. After 20 ml of ether was added, the cen-

trifuge tube was capped and stirred on a vortex mixer for 5 min, then centrifuged at about 2000 *g* for 5 min. With a Pasteur pipet, the clear supernate was transferred carefully into a 100-ml volumetric flask. The extraction was repeated four more times with 20-ml portions of ether. The combined ether extract was diluted to volume with ether, if necessary, and mixed. A 10-ml volume of this solution was pipetted into a 100-ml boiling flask and evaporated to dryness on the rotary evaporator. Ether traces were removed under a nitrogen stream and the residue was dissolved in 10 ml of water-saturated chloroform. The solution was transferred using a Pasteur pipet to the diatomaceous earth column, and the flow-rate was adjusted to 1–1.5 ml/min. The flask was rinsed twice with 20-ml portions of water-saturated chloroform and the rinsing were transferred to the column. The eluate was discarded.

The adsorbed saccharin was eluted with 50 ml of acetic acid–ethyl acetate–chloroform eluent, and the eluate was collected in a 100-ml boiling flask. The eluate was evaporated to dryness on the rotary evaporator at about 45°C. Acetic acid traces were removed under a nitrogen stream. The residue was dissolved in 5.0 ml (2000-ppm sample) or 3.0 ml (200-ppm sample) of the respective internal standard solution. The solution was filtered through a 5-μm membrane filter.

Chromatographic procedure

A 20-μl volume of the standard solution and 20.0 μl of the prepared feed sample solutions were chromatographed under the described HPLC conditions. The chromatogram was quantitated by relating the saccharin–sulfathiazole peak area ratio for the sample to that of the standard solution.

RESULTS AND DISCUSSION

Preliminary studies using a number of solvents and solvent mixtures indicated that the extractant of choice for saccharin was ether. Although saccharin is slightly soluble in ether, its concentration in the animal feed mix was sufficiently low so that multiple extractions could isolate the saccharin quantitatively. Furthermore, with ether the number of feed constituents co-extracting with saccharin was far less than if, for example, acetone was used in a direct liquid–solid extraction as described for the assay of azosemide in animal feed[10]. This resulted in cleaner chromatograms. The extraction with ether was done in the presence of hydrochloric acid so that the method is applicable for either saccharine or sodium saccharin in feed mix. Attempts to extract the compound using a shaker, separatory funnel, Soxhlet extractor, or ultrasonic mixing were unsuccessful; in these cases low recoveries were obtained.

The cleanup procedure was based on the acidic nature of saccharin. In the sodium hydroxide–diatomaceous earth column, saccharin was converted to its sodium salt. To ensure that this salt remained in the column water-saturated chloroform must be used. The sodium hydroxide strength for adsorption on diatomaceous earth and the total amount needed were determined. Under the conditions described the sodium hydroxide–saccharin mole ratio was about 300 and 30 for the 200- and 2000-ppm samples, respectively. Various eluents were investigated for optimum recovery of the trapped saccharin. This was performed by spiking ether extracts of a placebo feed mix with accurately known solutions of saccharin in ether and run through the cleanup steps. The proposed eluent mixture appeared to give optimum

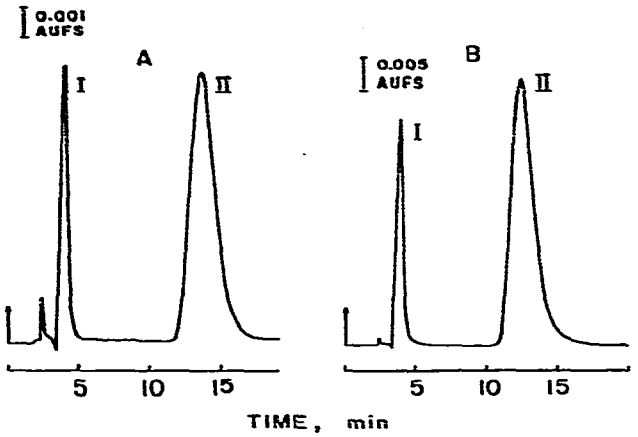

Fig. 1. Chromatograms of the appropriate saccharin (I) and sulfathiazole (II) standard solutions. A, at 0.02 a.u.f.s.; B, at 0.1 a.u.f.s.

recovery. In this mixture sufficient acetic acid was present to supply hydrogen ions which will exchange for the sodium ions of sodium saccharin thus rendering the saccharin in the free and elutable form. The addition of a bottom layer of plain diatomaceous earth in the column helped to break emulsions when they formed in the column.

Under the described HPLC conditions, saccharin and sulfathiazole eluted as symmetrical peaks and were very well resolved from one another. The retention times of saccharin and sulfathiazole were *ca.* 3.8 and 12.3 min, respectively (Fig. 1A and B). The average height equivalent to a theoretical plate (HETP) of the column ($\pm$ S.D.) was 1.116 $\pm$ 0.047 mm for saccharin (n = 10).

Quantitation was based on the drug–internal standard peak area ratio. With these ratios the linearity between detector response at 254 nm and amount of saccharin injected was established. Linearity was obtained between 0.4–5.0 μg of saccharin. A typical regression equation for the saccharin–sulfathiazole peak area ratio

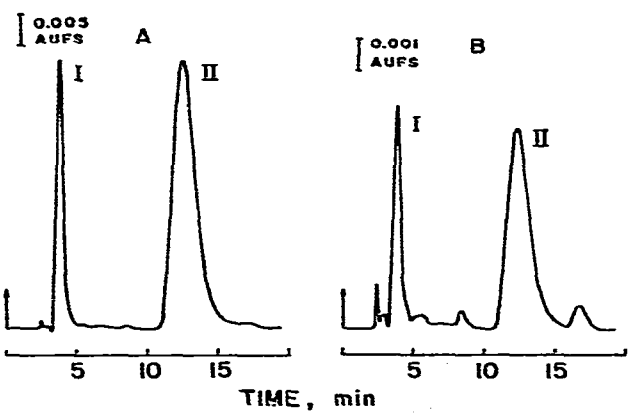

Fig. 2. Chromatograms of saccharin (I) from animal feed mix. II = sulfathiazole; A = 2000-ppm feed sample; B = 200-ppm feed sample.

(A) and amount of saccharin injected (C, expressed in μg) was $A = 0.467\ C + 0.044$ with a correlation coefficient $r = 0.9994$.

The cleanup procedure gave liquid chromatograms for the 2000-ppm sample that resolved saccharin and sulfathiazole from any feed constituents present (Fig. 2A). The liquid chromatograms for the 200-ppm sample showed some interference to the saccharin peak area by the feed constituents (Fig. 2B). This interference, however, amounted to less than 0.6%. For comparison, the liquid chromatograms of blank feed mix run under the conditions for the 200- and 2000-ppm samples, *i.e.* at 0.02 and 0.1 a.u.f.s., respectively, are shown in Fig. 3A and B.

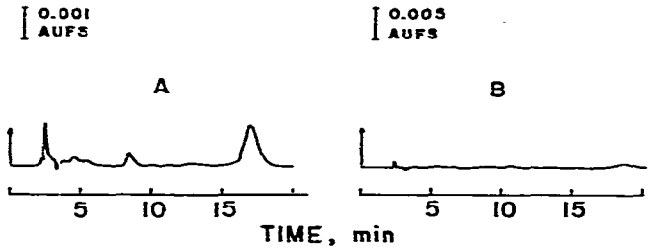

Fig. 3. Chromatograms of placebo feed mix. A, at 0.02 a.u.f.s.; B, at 0.1 a.u.f.s.

To provide better representation of the analytical potency, a concomitant standard was used, *i.e.* the standard was subjected to the same analytical steps in the cleanup procedure. This would eliminate any errors (losses) occuring during the cleanup steps.

Recovery studies were performed on feed samples spiked at 200- and 2000-ppm levels with saccharin or sodium saccharin. The accurately weighed drugs were carefully mixed using a mortar and pestle. Each spiked sample was about 50 g. Table I

TABLE I.

RECOVERY OF SACCHARIN FROM SPIKED FEED MIX AT 200- AND 2000-ppm LEVELS

Sample (ppm)	*Amount of saccharin*		*Recovery (%)*
	in analytical sample (mg)	*Found (mg)*	
200 ppm			
214	1.07	1.03	96.3
216	1.08	1.03	95.7
212	1.06	1.06	100.0
Overall recovery (%)			97.6
S.D.			2.1
2000 ppm			
2144	10.72	10.98	102.4
2152	10.76	10.58	98.3
2114	10.57	10.20	96.5
Overall recovery (%)			99.1
S.D.			3.0

shows the recovery data for saccharin from animal feed mix at the 200- and 2000-ppm levels. The overall percent recovery (± S.D., $n = 3$) were 97.6 ± 2.1% and 99.1 ± 3.0% for the 200- and 2000-ppm samples, respectively. The overall percent recoveries (± S.D., $n = 3$) for sodium saccharin from animal feed were 98.9 ± 2.1% and 100.4 ± 3.8% for the 200- and 2000-ppm samples, respectively (Table II).

TABLE II

RECOVERY OF SODIUM SACCHARIN FROM SPIKED FEED MIX AT 200- AND 2000-ppm LEVELS

Sample (ppm)	*Amount of sodium saccharin*		*Recovery (%)*
	in analytical sample (mg)	*Found (mg)*	
200 ppm			
288	1.44	1.44	100.0
286	1.43	1.38	96.5
289	1.45	1.46	100.1
Overall recovery (%)			98.9
S.D.			2.1
2000 ppm			
2900	14.50	14.60	100.7
2940	14.70	15.30	104.1
2880	14.40	13.90	96.5
Overall recovery (%)			100.4
S.D.			3.8

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