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Note

Quantitation of N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide and 2-amino-4-(5-nitro-2-furyl)thiazole in rodent diet

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Nitrofurans are one of the largest groups of heterocyclic nitro compounds available for human and veterinary medicinals and food additives[1]. A number of these agents are known to cause cancer in a variety of animal species[2]. Carcinogenesis is considered to be a multi-stage process and two separate events known as initiation and promotion are required for tumor production[3,4]. N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide (FANFT) is a potent carcinogen for rat bladder epithelium when fed in a diet at low levels[3–5]. It has been widely used as an initiator in studies of two-stage carcinogenesis using the rat bladder model[6–8].

Multi-stage carcinogenesis studies in our laboratory involve the feeding of diets containing FANFT, and a method for quantitative analysis has been developed. In addition, a high-performance liquid chromatography (HPLC) method has been devised for the determination of 2-amino-4-(5-nitro-2-furyl)thiazole (ANFT), which is a contaminant produced by deformylation of FANFT. These methods for quantitation of FANFT and ANFT in the diet allow for the calculation of ingested doses of carcinogen from food consumption data, as well as determination of the extent of FANFT decomposition during preparation, storage, and feeding of the diet.

EXPERIMENTAL

ANFT was a gift from Dr. S. Swaminathan (University of Wisconsin, Madison, WI, U.S.A.). FANFT was obtained from Dr. G. T. Bryan (University of Wisconsin, Madison, WI, U.S.A.) and purified by sublimation by Dr. D. S. Williamson. It was blended at a level of 0.2% (w/w) into various diets (Prolab 3200, Agway, St. Marys, OH, U.S.A., and NIH-07, Ziegler Bros, Gardners, PA, U.S.A.) for the proposed study. Spectral-grade solvents [dichloromethane, dimethylformamide (DMF), nitromethane and light petroleum (b.p. 35–60°C)] were obtained from Midland Scientific (Omaha, NE, U.S.A.). Silica gel (60–200 mesh) for column chromatography was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). All other materials were from commercial sources.

Pelleted diets were ground to a fine powder in a coffee grinder. Samples of powdered diet (*ca.* 1 g) were extracted with 5-ml aliquots of DMF and the insoluble material collected by centrifugation (10 min, 11 000 *g*). The supernatants were re-

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moved and stored. After four successive extractions with DMF, the pooled supernatants were adjusted to a final volume of 25 ml by addition of DMF.

A small quantity of glass wool was placed into a 3-ml syringe barrel as support for silica gel packing material (630 mg) which was lightly tamped down. The column was conditioned by washing with two 3-ml aliquots of dichloromethane prior to application of diet extract (300 μl) in DMF. The column was washed with 3 ml of light petroleum, and the nitrofurans eluted with nitromethane. This solution was adjusted to 5 ml with nitromethane and clarified by centrifugation (10 min, 11 000 *g*), prior to measuring the absorbance at 380 nm in a Perkin-Elmer Lambda 4B UV–VIS spectrophotometer. The amounts of nitrofuran present in the column eluate were determined by comparing the measured absorbance to a standard curve. The degradation of FANFT to ANFT was investigated by HPLC analysis of the DMF extract which was filtered (0.2-μm 13-mm Nylon Acrodisk, Gelman Sciences, Ann Arbor, MI, U.S.A.) prior to injection (10-μl aliquots). HPLC was performed on a Waters (Milford, MA, U.S.A.) system consisting of Model 510 pumps, a WISP 712 automated sample injector or U6K manual injector, temperature control unit and oven. A Waters end-capped C_{18} column (15 cm × 0.39 cm I.D., 4 μm particle size) was eluted isocratically at 65°C with 100 m*M* ammonium acetate–methanol (65:35), pH 6.0, at a flow-rate of 1.5 ml/min. The mobile phase was made using only HPLC-grade solvents and was filtered and degassed before use.

Nitrofurans were detected by using a Waters 481 UV–VIS spectrophotometer (380 nm; 0.001 a.u.f.s.) which was interfaced with a Hewlett-Packard Model 3390A integrator to provide on-line reporting of data. The absorbance wavelength was determined by use of a Perkin-Elmer Lambda 4B UV–VIS spectrophotometer. Fluorescence detection was also considered but use of the prescan facility on a Perkin-Elmer Model LS5B spectrofluorometer failed to provide data on excitation/emission maxima for the nitrofurans.

RESULTS

The standard curve for FANFT dissolved in nitromethane (10–60 μg/ml) was linear to 40 μg/ml (Fig. 1, top) and shows the upper limit of the assay. A second standard curve covering a lower range (0–13 μg/ml) is also linear (Fig. 1, bottom) and shows the lower limit for detection of FANFT by absorbance to be 1.64 mg/ml. The binding capacity of the silica gel solid-phase extraction column was determined by placing varying amounts (100–800 μl) of dietary extract (in DMF) on the columns (see Fig. 2). Binding was linear up to about 600 μl of extract so 300 μl of extract were applied to the solid-phase extraction column for routine analysis. The amount of nitrofuran in the NM extract was determined by interpolation from a standard curve constructed by solid-phase extraction of diet spiked with known amounts of FANFT. Extraction efficiency was determined by mixing 2 ml of FANFT (1 mg/ml in DMF) with 1 g of diet which was subject of extraction and chromatography as described above. The absorbance of the chromatographed standard was divided by that obtained from a non-chromatographed FANFT standard (4.8 μg/ml in nitromethane); under these conditions recovery was 95–101%. The percentage of nitrofuran in the diet was calculated by dividing the determined value by the weight of diet extracted. The assay was accurate providing determined values of 0.180% ± 0.014 when 16

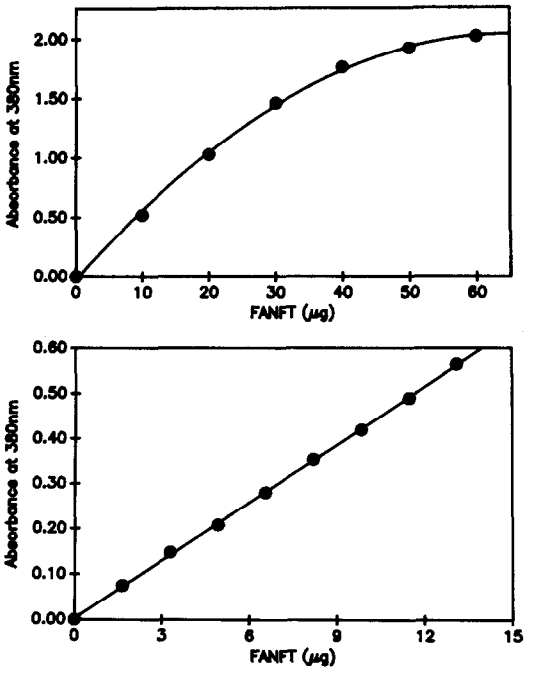

Fig. 1. Standard curves for FANFT in nitromethane. Top: 10–60 μg/ml; bottom: 0–13 μg/ml.

samples of diets containing 0.2% (w/w) FANFT were analyzed. Precision was 2.2% when one sample containing 0.2% (w/w) FANFT was subject to 10 repeat analyses (0.205 ± 0.005%). To determine the possible degradation of FANFT to ANFT, an HPLC method was employed since both nitrofurans exhibit maximum absorbance at 380 nm. The relative elution positions of FANFT and ANFT are shown in Fig. 3, and a standard curve for the quantitation of trace amounts of ANFT (5% or less of the total amount of nitrofuran injected) in FANFT is linear over the range 0–100 ng (Fig.

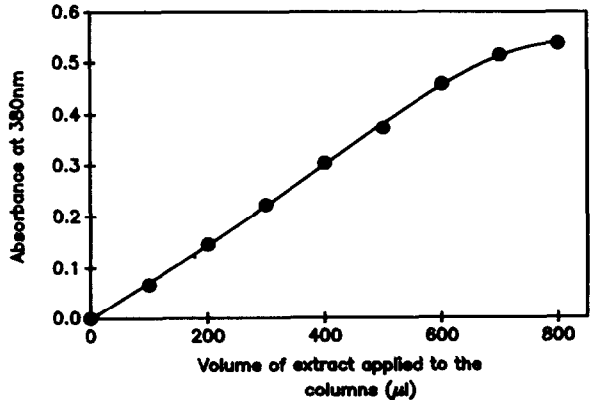

Fig. 2. Binding curve for DMF extract on the silica gel solid-phase extraction columns.

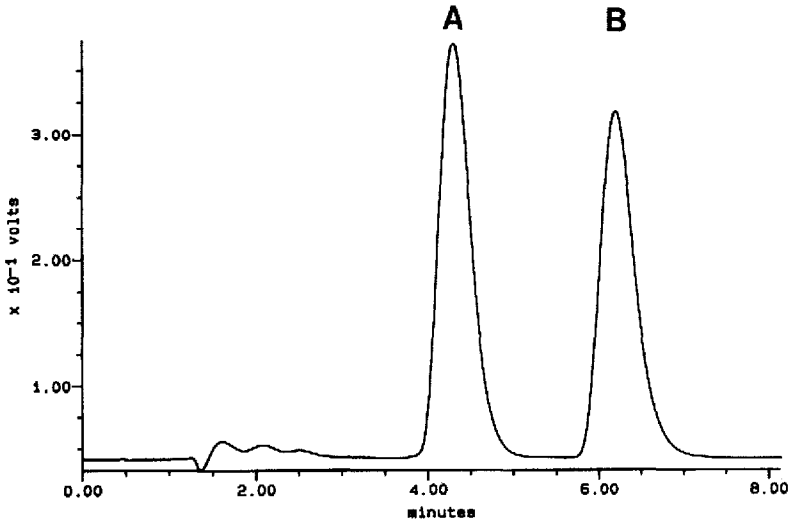

Fig. 3. HPLC analysis of a mixture of ANFT (peak A) and FANFT (peak B) to show relative elution position.

4). The lower limit for ANFT detection by HPLC was 4 ng and this amount corresponds to a contamination level of 0.5% when 10 μl of DMF extract of diet containing 0.2% FANFT are chromatographed.

This HPLC method was used to determine the stability of FANFT in two diets, Prolab 3200 and NIH-07 (Table I). The amount of ANFT in the DMF extract was determined from the standard curve (Fig. 4) and was expressed as a percentage of the total amount of nitrofuran in the extract which had been determined by solid-phase extraction and spectrophotometric assay at 380 nm (Fig. 1). The amount of FANFT slowly decreases up to 10 weeks after blending and the amounts are equivalent in each of the diets.

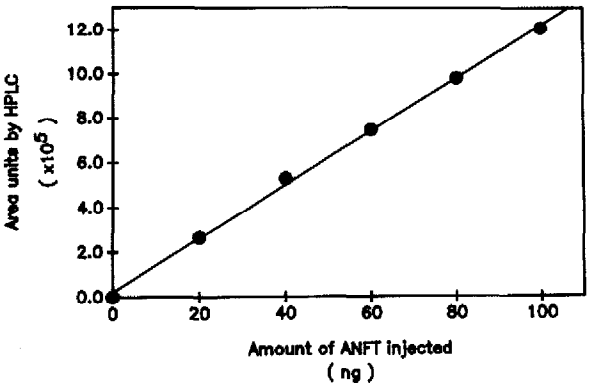

Fig. 4. Standard curve for the quantitation of ANFT (0–100 ng) in DMF extracts by HPLC. Each point is the mean of three injections.

TABLE I

HPLC ANALYSIS OF NITROFURANS IN RODENT DIETS AT VARIOUS TIMES AFTER BLENDING

Time (weeks)	*Diet*[a]			
	Prolab 3200		*NIH-07*	
	FANFT	*ANFT*	*FANFT*	*ANFT*
6	98.77	1.23	99.09	0.91
10	97.14	2.86	97.14	2.86

[a] Amounts are expressed as a percentage of total nitrofuran injected.

DISCUSSION

Nitrofurans have been assayed by microbiological[9] as well as chemical methods[9–16]. While the former are preferred for assessing potency they do not provide information on the purity of the agent and a chemical method is therefore preferred. Paper chromatography has been applied to the detection of many nitrofurans[10] but the technique is qualitative and a number of quantitative assays for specific nitrofurans exist. Column chromatography on cationic Amberlite resin has been used in the isolation of nifurpipone from urine prior to quantitation by colorimetric or spectrophotometric methods[11]. Similar methods involving solvent extraction and formation of a chromophore have been reported for nitrofurantoin[12–14]. An HPLC method for nitrofurantoin requires solvent extraction and rotary evaporation prior to analysis on an octylsilane column[15].

This method for the rapid extraction and sensitive quantitation of FANFT and its deformylated derivative, ANFT, is based upon published methods[16]. The extremely low solubility of FANFT in aqueous media requires the use of an organic solvent, DMF, for the initial extraction prior to solid-phase extraction on silica gel columns. The wash with light petroleum during this adsorption chromatography allows for the removal of unknown contaminants which are soluble in DMF but not NM.

The quantitation of FANFT is based on the absorbance of the NM eluate at 380 nm and provides a rapid method for determining the amount of FANFT in a diet sample. Derivatization before detection was not necessary due to the strong absorbance of the nitro group at this wavelength. However, FANFT deformylates to ANFT, which also absorbs at 380 nm and this spectrophotometric technique does not distinguish between the two nitrofurans. An HPLC method was, therefore, devised to quantitate trace amounts of ANFT ($<5\%$ of the total amount of nitrofuran) in the presence of FANFT. This was achieved by adapting a published procedure[16] and increasing column temperature to 65°C and the flow-rate to 1.5 ml/min. This elevated temperature resulted in sharper peak profiles with reduced retention times and was found to greatly facilitate detection of ANFT in the nanogram range at high detector sensitivities (0.001 a.u.f.s.). Direct injection of a filtered DMF extract onto the HPLC allows for rapid quantitation of ANFT in an extract and is useful in determining the stability of FANFT in diets as well as the degree of contamination by ANFT. These data show that FANFT is stable in both diets (Table I) and that the diets contain equivalent amounts of ANFT at each time point.

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