Journal of Chromatography, 280 (1983) 394–399
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,193

Note

Quantitative determination of ethylenediamine in a laboratory rodent diet by high-performance liquid chromatography

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(First received May 11th, 1983; revised manuscript received August 2nd, 1983)

Ethylenediamine (EDA) is a major industrial chemical with a wide variety of applications. Since 1979, a comprehensive toxicology program has been in progress at our laboratory under the joint sponsorship of Union Carbide Corporation, Dow Chemical Company and Texaco Chemical Company for the safety evaluation of this chemical. Some of the studies in this program are being reported elsewhere[1–4].

In order to conduct animal chronic toxicity studies via dietary exposure to animals, development of an analytical technique is necessary to ensure accurate incorporation of various levels of ethylenediamine dihydrochloride (EDA · 2HCl) into the diet. At least two reports have been published on the high-performance liquid chromatographic (HPLC) analyses of di- and polyamines[5,6] but both involved lengthy incubation periods and solvent evaporation processes. The present paper describes an HPLC method for the quantitative determination of EDA · 2HCl in the laboratory rodent diet. A large portion of the work was also devoted to finding a suitable solvent for the extraction of EDA · 2HCl from the diet.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of Waters Model 6000A solvent delivery systems, a Model 660 solvent programmer, a U6K injector, a Model 440 absorbance detector and a 420E fluorescence detector. The chromatogram was traced on an Omniscribble Model A5211-1 dual-pen chart recorder (Houston, TX, U.S.A.).

Materials

EDA · 2HCl used in this study was the same sample used for our toxicology program. This sample was synthesized and supplied by Union Carbide Corporation, Chemicals and Plastics Division, South Charleston, WV, in July, 1979. On the basis of various analyses (elemental, water, infrared spectroscopic, emission spectrographic and HPLC analyses), this EDA · 2HCl sample was considered to be of high purity with no detectable impurities. [1,2-^{14}C] EDA · 2HCl (specific activity 8.0 mCi/mmole) was purchased from New England Nuclear, Boston, MA, U.S.A. The radiochemical

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0021-9673/83/$03.00

purity was established to be greater than 99% by paper chromatography. The chemical identity was reconfirmed by mass spectrometry. High-purity solvents (distilled in glass) were obtained from Burdick and Jackson Lab., Muskegon, MI, U.S.A. 5-Dimethylamino-1-naphthalenesulfonyl chloride (Dns Cl) was ordered from Sigma Chemical Company, St. Louis, MO, U.S.A. The diet was NIH-07 rodent diet from Ziegler Brothers, Gardners, PA, U.S.A.

Extraction of EDA · 2HCl from diet samples

A 20-ml amount of extracting solvent (de-ionized water or dilute HCl) was added to 5 g of EDA · 2HCl-containing diet samples in a 35-ml screw-capped centrifuge tube. The mixture was stirred and then vigorously mixed for 30 sec by a Brinkmann Polytron Homogenizer. The tube was then capped and centrifuged for 15 min at 42,000 *g* in an International Model B-20A centrifuge. The supernatant was poured into a bottle, capped and stored in the refrigerator for further analysis.

Dansylation reaction

The dansylation procedure was a modification of that by Newton *et al.*[5]. The dansylation reaction was carried out in 20-ml liquid scintillation counting vials. In each vial, 100 μl each of water, diet extract (or standard solution), 0.3 *M* $HClO_4$, saturated $NaHCO_3$ and 600 μl of Dns Cl in acetone (70 mg/ml) were added in sequential order. The vials were covered and shaken gently in a 50°C water bath for 1 h. At the end of the reaction 5 ml of toluene were added to each vial to extract the dansylated EDA from the aqueous layer. The capped vials were vortexed twice for 30 sec and stored in a freezer overnight. The next morning, when the vials were warmed to room temperature, they were vortexed again (2 × 30 sec). Then the mixture was transferred to a Corex No. 8441 centrifuge tube and centrifuged for 3 min at 2200 *g*. The toluene layer was transferred with a Pasteur pipette and analyzed by HPLC.

HPLC analysis

Five μl of the toluene extract of the dansylated EDA were injected onto a Waters Bondapak CN-10 column for analysis at ambient temperature. A programmed solvent gradient elution[5] was carried out with two solvents: Solvent A consisted of cyclohexane–isopropanol (49:1, v/v), and Solvent B consisted of cyclohexane–methylene chloride–isopropanol (21:3:1, v/v). The sample was first eluted in the isocratic mode with equal amounts of Solvent A and B for 2 min, and then eluted by the concave gradient curve number 7. The gradient changed from 50 to 100% B in 5 min at a flow-rate of 3 ml/min. Depending on the column condition, the maximum pressure developed during the runs was about 3500 p.s.i. The fluorescence detector was activated at 340 nm with an emission filter at 425 nm. The speed of the chart recorder was set at 0.5 cm/min.

Recovery experiments

The quantitative recovery of EDA · 2HCl from the rodent diet was conducted by two approaches: recovery with respect to mass of EDA · 2HCl, and recovery with respect to radioactivity of [^{14}C]EDA · 2HCl.

Recovery with respect to mass. The series of diet samples containing a known

amount of EDA · 2HCl were extracted by the testing solvents. This was followed by dansylation and HPLC analysis. The recovery rates were calculated from a standard curve prepared from a series of direct standards (EDA · 2HCl water solutions used directly for analysis).

Recovery with respect to radioactivity. A series of diet samples containing a known amount of [^{14}C]EDA · 2HCl were extracted by the testing solvents. The radioactivity of the extracts was counted to obtain the percentage recovery.

Application to diet analysis

The method described above was employed to determine the EDA · 2HCl concentrations in more than 174 batches of test diet associated with various toxicity studies including chronic toxicity studies. In addition, the homogeneity and stability of EDA · 2HCl in the test diets were investigated. The experimental procedures for these studies are briefly described below.

At monthly intervals, two coded diet samples were selected from the biweekly preparation of the test diet. These samples and appropriate standards were then extracted and analyzed as described above. If the analytical results obtained for any sample deviated by ±10% from the calculated value, that particular sample was reanalyzed.

The homogeneity of the test diet was studied a total of three times to check the suitability of the diet preparation method. Sampling of a test diet at 5 positions in the mixing bowl (North, East, Center, South, West) was carried out immediately following the preparation of a batch of test diet. To investigate the stability of EDA · 2HCl in the test diet, analyses were made on the same sample which was kept at room temperature over a six-month period. The sample was analyzed at 0 (the day of preparation), 2, 5, 8 and 14 days and at 1, 2, 3, 4, 5 and 6 months.

RESULTS AND DISCUSSION

Typical chromatograms of the dansylated EDA derivatives are shown in Fig. 1. The retention time of the dansylated EDA under the experimental conditions was about 10 min. It was separated from other interfering substances in the diet. Under similar experimental conditions, the chromatograms of the control diet showed all the other peaks in Fig. 1B except the Dns-EDA peak. Thus, it is apparent that there were no biogenic amines in our rodent diet as reported to be present in other foodstuffs[7]. Accordingly, this method is specific for EDA analysis in the laboratory rodent diet. The peak height of dansylated EDA was recorded and the concentration of EDA · 2HCl in the diet was interpolated from a standard curve which was constructed by points covering the range of the unknowns.

At the beginning of this investigation, water was used as a solvent for extracting EDA from the diet. It was found that the recovery was low. Consequently, diluted NaOH and HCl were tested for their suitability as extracting solvents. While the results from the diluted NaOH were unsatisfactory, those from the diluted HCl were promising. Table I is a summary of the results from diluted HCl; for comparison, the data from water extraction are also included. It is apparent that 0.25 *M* HCl is the solvent of choice. Subsequent experiments using this solvent (0.25 *M* HCl) resulted in a recovery rate between 83 and 95% over a wide range of concentrations

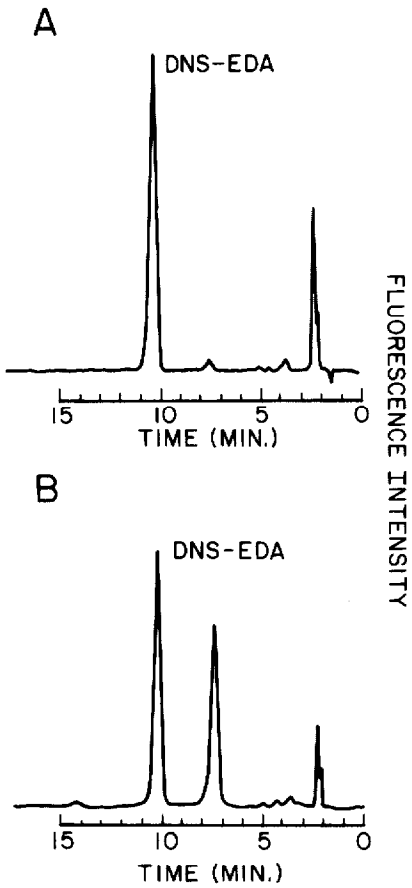

Fig. 1. High-performance liquid chromatograms of dansylated EDA (Dns-EDA) from (A) an EDA standard, (B) a test diet extract.

TABLE I

RECOVERY OF EDA · 2HCl FROM THE RODENT DIET WITH RESPECT TO MASS

Sample	*Solvent*	*pH of extract*	*EDA · 2HCl added (mg)*	*EDA · 2HCl recovered (mg)*	*Recovery* (%)*
1	water	5.58	24.0	12.1	50.4
2	0.10 *M* HCl	3.97	24.0	17.2	71.7
3	0.20 *M* HCl	2.61	24.0	18.0	75.0
4	0.25 *M* HCl	2.03	24.0	21.2	88.3
5	0.50 *M* HCl	1.15	24.0	20.0	83.3
6	1.00 *M* HCl	0.61	24.0	0	0

* Values are average of two experiments.

TABLE II

EXAMPLES OF EDA · 2HCl CONCENTRATION DETERMINATIONS OF TEST DIET SAMPLES

*Sample code number**	*EDA · 2HCl calculated (%)*	*EDA · 2HCl found*** *(%)*
T-081479-244	0.42	0.39
T-082979-151	0.027	0.027
T-102479-470	7.00	7.68, 6.94
T-112179-611	0.041	0.038
T-012980-468	0.218	0.212
T-042280-832	0.712	0.699
T-081280-210	0.863	0.960, 0.880
T-120180-906	0.446	0.436
T-031281-904	5.0	4.6
T-041381-224	0.115	0.124

* All samples were analyzed blind.

** Where there were more than one analysis, all values are presented.

of EDA · 2HCl-containing diet. In other recovery studies using [^{14}C]EDA · 2HCl-containing diet samples, the extraction rate (calculated, based on radioactivity) by 0.25 *M* HCl was shown to be quantitative. The less than quantitative recovery obtained when recovery was calculated based on mass was therefore due to the less than quantitative yield of dansylation reaction.

For our routine work, diet samples usually included a wide range of EDA · 2HCl concentrations. Two standard curves with slightly different slopes were used: a low-concentration standard curve covered the range 0–10 mg per 5 g diet, and a high-concentration standard curve covered the range 10–50 mg per 5 g diet. Under these conditions, the linearity of each standard curve was excellent (*i.e.* correlation coefficient for linear regression analysis higher than 0.99). Table II contains some representative analyses from more than 174 batches of test diets. The analytical results for low concentrations of EDA · 2HCl in the diet were in general very good, while those for high concentrations showed a little more variability. Diluted extract was used to analyze the samples with the highest concentrations. Tables III and IV are summaries of the homogeneity and stability studies. As the data illustrate, the distribution of EDA · 2HCl in the diet was homogeneous (Table III) and EDA · 2HCl was stable in the diet for up to 6 months (Table IV).

TABLE III

HOMOGENEITY STUDY — EDA · 2HCl DISTRIBUTION IN A TEST DIET PREPARATION

Calculated concentration of EDA · 2HCl: 0.50%.

Position sampled in the mixing bowl	*Found in the diet (%)*
North	0.48
East	0.48
Center	0.49
South	0.48
West	0.51
Mean ± standard deviation	0.49 ± 0.01

TABLE IV

STABILITY OF EDA · 2HCl IN A TEST DIET PREPARATION

Calculated concentration of EDA · 2HCl: 0.60%.

Day of study	*Found in the diet (%)*
0*	0.62
2	0.62
5	0.65
8	0.60
14	0.60
30	0.59
61	0.60
92	0.59
126	0.56
159	0.59
183	0.61
Mean ± standard deviation	0.60 ± 0.02

* Zero refers to the day the diet was prepared.

In conclusion, an HPLC method was developed for the quantitation of EDA in EDA · 2HCl-containing rodent diet samples. The method is reproducible and accurate. Furthermore, it provides the flexibility of much shorter analysis time. The most suitable solvent for extracting EDA from the diet samples was found to be 0.25 *M* HCl. These techniques should be readily adaptable to EDA analyses in other samples of biological significance.

REFERENCES

1 R. S. H. Yang and M. J. Tallant, *Fundam. Appl. Toxicol.*, 2 (1982) 252.
2 R. S. H. Yang, R. H. Garman, R. R. Maronpot, J. A. McKelvey, C. S. Weil and M. D. Woodside, *Fundam. Appl. Toxicol.* (1983) in press.
3 R. S. Slesinski, P. G. Guzzie, W. C. Hengler, P. G. Watanabe, M. D. Woodside and R. S. H. Yang, *Mutation Res.*, (1983) in press.
4 R. S. H. Yang, R. H. Garman, E. V. Weaver and M. D. Woodside, *Fundam. Appl. Toxicol.*, (1984) in press.
5 N. E. Newton, K. Ohno and M. M. Abdel-Monem, *J. Chromatogr.*, 124 (1976) 277.
6 N. Seiler, B. Knödgen and F. Eisenbeiss, *J. Chromatogr.*, 145 (1978) 29.
7 N. Sayem-El-Daher, R. E. Simard, L. l'Heureux and A. G. Roberge, *J. Chromatogr.*, 256 (1983) 313.