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DETERMINATION OF ACRYLAMIDE IN NERVE TISSUE HOMOGENATES BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

Acrylamide in biological samples can be determined by gas chromatography with electron-capture detection after conversion to its 2,3-dibromopropionamide derivative. The derivatization is carried out in aqueous solution, plasma or tissue homogenates by ionic bromination and the reaction conditions and sample clean-up are described. The detection limit corresponds to $9.5 \cdot 10^{-12}$ g of acrylamide on column or $8.4 \cdot 10^{-9}$ g in the final biological extract (0.5 ml). The overall recovery of acrylamide spiked samples at the nanogram level exceeds 80%. It was found that the accumulation of free acrylamide in the sciatic nerve distal region of rats intoxicated with acrylamide was less than 2–8 ppm.

INTRODUCTION

Acrylamide monomer is made on a large scale for the manufacture of a wide range of polyacrylamide products. The polymer itself is considered to be non-toxic but the monomer has been demonstrated to be a potent cumulative neurotoxin in animals and man[1]. Occupational exposure to acrylamide monomer in subtoxic doses can lead to truncal ataxia and peripheral neuropathy causing drowsiness, a lack of concentration, difficulty in using the extremities and a loss of sensation distally in the limbs. At higher concentrations, sensorimotor neuropathy, encephalopathy with confusion, disorientation, memory disturbances and hallucinations may occur. Since the monomer but not the polymer is neurotoxic, it is mainly those involved in the manufacture of the monomer or in the polymerization process that are most at risk in developing toxic effects. The general population is exposed to the waste products of the polyacrylamide industries as well as residual monomer contained in the finished product. For example, up to 2% of residual monomer is allowed in polyacrylamide flocculators for general industrial use. Polyacrylamide used as a strengthener for paper in contact with foodstuffs or for purification of drinking water is permitted to

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contain not more than 0.05% (w/w) of monomer. The safe limit for airborne exposure to acrylamide monomer adopted by the American Conference of Government Industrial Hygienists in 1971 was 0.3 mg m^{-3} (ref. 2) and it was assessed that the total daily occupational exposure to acrylamide monomer should not exceed 0.05 mg kg^{-1} body weight[3].

Analytical methods for determining acrylamide include colorimetry[4], gas chromatography (GC)[5–7], high-pressure liquid chromatography[8–10] and differential pulse polarography[11,12]. Acrylamide reacts with diazomethane in a methanol–ether solution to form a pyrazoline derivative which can be reacted with 4-dimethylaminocinnamaldehyde to form a brightly colored purple Schiff base complex[4]. This reaction is not specific for acrylamide (a general reaction for amides and esters) and is fairly insensitive for environmental applications. The analysis of acrylamide by gas chromatography with flame-ionization detection also lacks the selectivity and sensitivity required for environmental applications[5,13]. Acrylamide can be converted to its 2,3-dibromopropionamide derivative by UV-light catalysed free radical bromination[6] or in an improved reaction by ionic bromination[7] for use with the electron-capture detector (ECD). For waste water, as little as 0.03–0.1 $\mu g\ l^{-1}$ of acrylamide as its 2,3-dibromopropionamide derivative could be detected. The non-specific UV detection of acrylamide after separation by high-pressure liquid chromatography could determine 0.1 mg l^{-1} (refs. 11, 12) or as the 2,3-dibromopropionamide derivative (after a sample concentration procedure), 0.2 $\mu g\ l^{-1}$ in water samples[8]. Differential pulse polarography can be used to determine acrylamide in dust and airborne samples by particle filtration and vapor collection in a water impinger with a sensitivity of 0.5 $\mu g\ ml^{-1}$ in the final extract.

The GC methods employing the 2,3-dibromopropionamide derivative and the selective and sensitive ECD are the most suitable for trace level analysis of acrylamide in environmental and physiological samples. In this paper, we have investigated and eliminated some of the inconsistencies in the published procedures[6–8] which should make the analysis of acrylamide by GC–ECD less troublesome. The working procedure is demonstrated for the analysis of plasma spiked with acrylamide and for the detection of acrylamide in the proximal and distal regions of excised sciatic nerves from rats administered acrylamide subcutaneously.

EXPERIMENTAL

Pesticide grade methanol, ethyl acetate and analytical grade hydrobromic acid (47–49%, v/v, water), sodium thiosulfate, sodium sulfate and potassium bromide were obtained from J. T. Baker (Arlington, TX, U.S.A.). Acrylamide monomer was electrophoresis-reagent grade from Eastman (Rochester, NY, U.S.A.). Anhydrous sodium sulfate was pretreated by heating overnight at 600°C. A saturated solution of bromide water was prepared by shaking distilled water with excess bromide and allowing the solution to stand for 1 h in a refrigerator at 4°C. The aqueous phase was removed as required for the derivatization reaction. An authentic sample of 2,3-dibromopropionamide (m.p. 132–133°C) was prepared as described by Hashimoto[7].

Biological samples

Plasma. Plasma was obtained from the blood bank and spiked with acrylamide prior to analysis using the procedure described for tissue samples.

Nerve tissue. The samples analyzed form part of a broader study to determine the effect of dietary pyruvate on acrylamide neurotoxicity. Male Sprague–Dawley rats were injected daily (5 days per week) subcutaneously with acrylamide (35 mg kg^{-1}) for periods of 0 to 4 weeks. Diet was not restricted and some rats received additional pyruvate as a 2% (w/w) sodium pyruvate supplement to their normal food. The rats were killed, the proximal and distal regions of the sciatic nerve removed and homogenized in a small volume of sucrose. The final sample contained approximately 1–5 mg of nerve tissue in 0.5 ml of sucrose solution.

Procedure for analyzing tissue samples

To a glass-stoppered flask (250 ml) was added distilled water (50 ml, double distilled from acid permanganate), 0.5 ml of the rat tissue homogenate, potassium bromide (7.5 g), a magnetic stirring bar and sufficient hydrobromic acid to adjust the pH to between 1 and 3. The flask was wrapped in aluminum foil to exclude light and saturated bromine water (2.5 ml) was added with stirring. The reaction was then allowed to proceed for 2 h in the dark at 0°C, excess bromine decomposed by dropwise addition of sodium thiosulfate solution (1.0 *M*) and sodium sulfate (15 g) added with vigorous stirring. The reaction mixture and washings (2 × 1.0 ml distilled water) were transferred to a separatory funnel and extracted with 15 ml and 10 ml of ethyl acetate. The organic phase was dried with anhydrous sodium sulfate, centrifuged and the extract and rinsings (2 × 1.0 ml ethyl acetate) evaporated to 0.50 ml under a gentle stream of nitrogen at 67°C. An aliquot of 2.0 μl was injected into the gas chromatograph from the final extract.

Gas chromatography

For GC, a Tracor 560 GC (Austin, TX, U.S.A.) with a constant-current pulse-modulated Ni^{63} ECD and a Hall 700 A electrolytic conductivity detector was used. Using the ECD, separations were carried out on a 6 ft. × 2.0 mm I.D. glass column packed with 5% (w/w) FFAP on Gas-Chrom Q (100–120 mesh), argon–methane (95:5) carrier gas flow-rate 35 ml min^{-1}, column temperature 155°C, injector temperature 180°C and detector temperature 350°C. The Hall conductivity detector was operated with a 6 ft. × 2.0 mm I.D. glass column packed with 1.5% OV-225 on Gas-Chrom Q (100–120 mesh) in the halogen mode with *n*-propanol at 0.4 ml min^{-1} as the support electrolyte. The injector temperature was 180°C, column temperature 145°C, reactor base temperature 200°C, nickel catalyst reactor temperature 850°C, and the hydrogen reduction gas flow-rate 40 ml min^{-1}. Helium was used as carrier gas at 30 ml min^{-1}.

RESULTS AND DISCUSSION

The ionic bromination of acrylamide in aqueous systems is described in refs. 6–8. We can confirm the observations of these workers and have extended the reaction to include samples from biological systems. The overall recovery of acrylamide as its 2,3-dibromopropionamide derivative is in the range 80–90% for nanogram amounts (*i.e.*, 10–1000 ppb concentrations) in aqueous solution or biological samples (Table I). For the same concentration range, the extraction efficiency of the solvent partition system was 96–100%, indicating that the principal loss of acrylamide occurs at the

TABLE I

OVER-ALL RECOVERY OF ACRYLAMIDE MONOMER AS 2,3-DIBROMOPROPIONAMIDE

Sample volume = 0.5 ml, pH = 1.6.

Sample	*Acrylamide monomer spiked (μg)*	*Amount of 2,3 DBPA* (μg)*		*Over-all recovery acrylamide monomer (%)*	*Relative standard deviation (%)*
		Calculated	*Found***		
Distilled water	0.04816	0.15668	0.13165	84.0	0.5
	0.04816	0.15668	0.13332	85.1	1.8
	0.02408	0.07834	0.06993	89.3	3.3
Plasma	0.08896	0.28943	0.26251	90.7	1.1
	0.04448	0.14471	0.12561	86.8	1.2

* 2,3-Dibromopropionamide.

** Procedure described in Experimental section. Each value is the mean of three determinations.

derivatization stage (the ionic bromination does not go to completion) or is due to adsorption of the derivative on the walls of the transfer vessel, etc. and at the extraction solvent evaporation stage. The use of an air stream as an evaporation aid, temperatures in excess of 80°C and evaporation of the extracting solvent to a residue prior to reconstituting the extract in fresh solvent all gave rise to a low recovery of derivative. For spiked samples at the μg level, evaporating the solvent to a residue at 70°C under nitrogen and then redissolving the sample in distilled water, led to a loss of 70% of the original 2,3-dibromopropionamide derivative. In agreement with others[6–8], it is important that the ionic bromination of acrylamide be performed at a low pH (pH 1–3 is optimum, acrylamide is rapidly hydrolyzed at basic pH), that a large amount of salting-out agent be employed at the extraction stage (2,3-dibromopropionamide has appreciable water solubility) and that the derivative at all times be protected from extremes of heat or light. Following the protocol outlined in the experimental section, recoveries in excess of 80% for the original acrylamide present in aqueous solution and spiked samples were consistently obtained and this was considered adequate for our purposes without further investigation.

A great deal of care is required in the GC analysis of 2,3-dibromopropionamide. Decomposition of the derivative occurred on columns of Carbowax 20M and STAP at temperatures of 150°C and on nickel columns in general. Silanized glass columns are required for this analysis. Non-polar columns such as OV-1 showed little retention of the derivative and glass columns packed with 1.5% (w/w) of OV-225 or 5% (w/w) FFAP on an inert support such as Gas-Chrom Q gave the best results. The FFAP column provided separation of the 2,3-dibromopropionamide derivative from co-extractants in biological fluids and was used for the analysis of these samples. At temperatures above 180°C, evidence for the thermal decomposition of the 2,3-dibromopropionamide derivative was found. The injection temperature and column temperature should be maintained below this value for all analyses.

At the onset of this study, we wished to take advantage of the halogen specificity and detector sensitivity of the Hall electrolytic conductivity detector for the

determination of 2,3-dibromopropionamide. However, the minimum detectable quantity of the derivative under optimum analytical conditions was found to be approximately 100 ng on-column. This was not adequate for our purposes but is in keeping with our general observation that, in the halogen mode, the Hall detector is approximately 10^3 times more sensitive to chlorine-containing than bromine-containing compounds. It was also noted with the Hall detector that if the reactor base temperature was set higher than 210°C, then a very dramatic loss of response to the derivative occurred. For these reasons, the less specific ECD was chosen for this analysis.

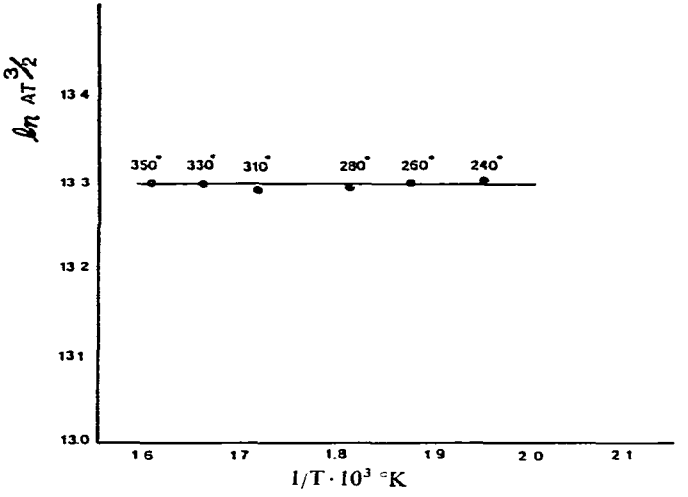

Fig. 1. Temperature dependence of the ECD response to acrylamide plotted as ln $AT^{3.2}$ vs. $1/T$ (A = peak area for a fixed mass of acrylamide, T = detector temperature in °K).

The ECD was found to provide adequate sensitivity and selectivity for the determination of the 2,3-dibromopropionamide derivative in biological extracts. Acrylamide itself is a weakly non-dissociative (Fig. 1) electron-capturing compound with a detection limit of 15.3 ng at 300°C (the detection limit is little influenced by detector temperature). The 2,3-dibromopropionamide derivative captures electrons by a dissociative mechanism (Fig. 2) and had a detection limit of 9.5 pg (as acrylamide) with a detector temperature of 350°C. The detector response was linear over the concentration ranges investigated, 10–100 pg and 50–170 pg. The operation of the ECD at 350°C, as well as maximizing the response of the detector to the derivative and reducing downtime due to cleaning, also usefully suppresses the response of certain unidentified co-extractants from the nerve tissue samples and reduces the width of the solvent front, giving a much cleaner-looking chromatogram.

The pathological substrate of acrylamide is distal, retrograde degeneration of long nerve fibers supplying the affected regions. Prior to onset of signs and symptoms, distal nerve fibers accumulate materials that are normally transported along the nerve fiber axon. This early abnormality in axonal transport raised the possibility that acrylamide affected energy pathways —on particular, glycolysis, in which the axonal

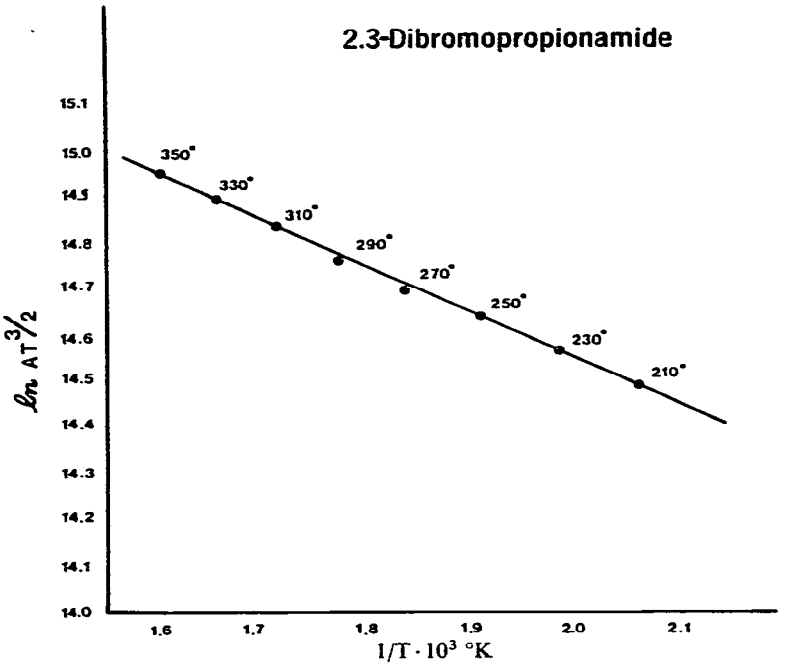

Fig. 2. Temperature dependence of the ECD response to 2,3-dibromopropionamide. Other conditions as in Fig. 1.

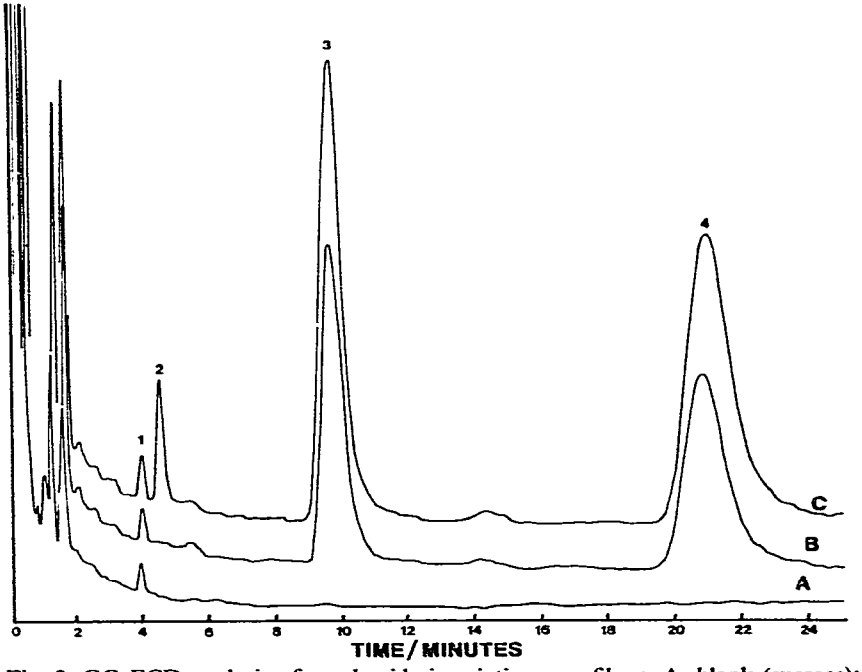

Fig. 3. GC–ECD analysis of acrylamide in sciatic nerve fibers. A, blank (sucrose); B, sample with no acrylamide; C, sample containing acrylamide. Peaks: 1 = impurity in blank; 2 = 2,3-dibromopropionamide; 3 and 4 = tissue co-extractants.

transport system depends. The purpose of this study was to test the hypothesis that acrylamide is the causative agent of axonal disintegration, will be accumulated in the nerve fibers, and its effect can be inhibited by providing pyruvate to the intoxicated rat (pyruvate should be capable of providing an alternative source of ATP to drive the axonal transport system). This hypothesis is supported by the physiological evidence accumulated during this study and will be published elsewhere. The chemical evidence does not support an accumulation of free acrylamide in the distal nerve endings. Of twenty-two nerve fiber samples analyzed, twenty of these contained less than $8.4 \cdot 10^{-10}$ g of acrylamide and the remaining two $5.16 \cdot 10^{-9}$ g and $1.53 \cdot 10^{-9}$ g of acrylamide. No distinction between the accumulation of acrylamide in the nerve fiber of rats receiving a dietary pyruvate supplement and those without was possible. Fig. 3 shows a composite chromatogram of a sucrose blank, a nerve sample without acrylamide and a sample found to contain acrylamide. At present, no particular weight is being assigned to the finding of acrylamide above the detection limit in the two samples as the origin may be contamination rather than biological accumulation (one sample came from a rat on pyruvate treatment, the other not receiving pyruvate). The conclusion to be drawn from this study is that the accumulation of free acrylamide in the damaged nerve tissues of acrylamide intoxicated rats is less than $8.4 \cdot 10^{-9}$ g (in samples of 1–5 mg). The emphasis is placed on "free acrylamide", as chemically attached acrylamide would not necessarily be detected by this method. Further studies are in progress to gain a better understanding of how acrylamide is able to attack selectively the distal nerve system and bring about its degeneration.

CONCLUSIONS

A very sensitive method has been developed for the determination of acrylamide as its 2,3-dibromopropionamide derivative in biological samples by GC–ECD. The detection limit corresponds to 9.5 pg on-column or $8.4 \cdot 10^{-9}$ g (allowing for the overall recovery) of acrylamide in biological fluids. Rats intoxicated with acrylamide were found to accumulate less than 2–8 ppm of acrylamide in their distal sciatic nerve fibers.

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