Journal of Chromatography, 518 (1990) 385–389
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 665

Note

Derivatization–liquid chromatographic assay of chloroacetaldehyde in biological samples

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(First received January 17th, 1990; revised manuscript received June 29th, 1990)

In connection with ongoing work in our laboratory, we desired a quantitative method for assaying chloroacetaldehyde (CA), a metabolite of drugs containing NCH_2CH_2Cl moieties. While several techniques have been employed to detect chloroacetaldehyde in biologic media, only three provide a means of quantitative assay. One of these, in which CA was derivatized with 2,4-dinitrophenylhydrazine followed by thin-layer chromatographic analysis of the resulting hydrazone, has served in the case of radio-labelled CA [1]. Alternatively, fluorimetric detection of $1,N^6$-ethenoadenine, an adduct formed from CA and cyclic AMP, has been used to measure CA [2]. Finally, gas-liquid chromatographic techniques have been used, coupled with both flame ionization [3] and electron-capture [4,5] detection to quantify CA in biological samples [3–5].

While these methods constitute sensitive and useful approaches our aim was to develop a convenient yet reliable high-performance liquid chromatographic (HPLC) assay requiring only standard UV detection while providing good sensitivity and linearity in the low nmol/mL concentration range. We also hoped to circumvent the need for liquid–liquid extractions, which become cumbersome in the case of multiple samples, and the need for synthesizing standards. This paper describes a novel assay for CA, utilizing the well-established cyclization reaction of CA with thiourea [6,7] to "trap" CA as 2-aminothiazole (2AT; Fig. 1). By virtue of its primary amino group, 2-AT may be readily concentrated by sorption on cation-exchange resins. It also exhibits an absorption max at 255 nm, enabling UV detection following HPLC. The application of our procedure to CA contained in water and in rat liver microsomal (RLM) suspensions is discussed.

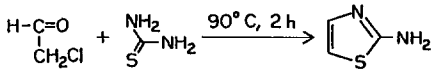

Fig. 1. Reaction of chloroacetaldehyde with thiourea to form 2-aminothiazole.

0021-9673/90/$03.50

EXPERIMENTAL

All solvents used were of HPLC grade (J. T. Baker, Phillipsburg, NJ, U.S.A.). Water was distilled and deionized using a Continental Water Systems Type 1 purification system. Prepared mobile phase was passed through a 0.45-μm nylon 66 filter and degassed by sonication. 2-AT (97%, Aldrich, Milwaukee, WI, U.S.A.) was recrystallized from, benzene–light petroleum (b.p. 35–60°C) prior to use. Chloroacetaldehyde (50%, w/w, solution in water, Aldrich) was verified for CA content (by titration [8]) and used as received. All other chemicals were of reagent grade.

Preparation of stock solutions

Aqueous stock solutions of CA and thiourea were prepared by dilution of the 50% CA solution to a final concentration of 200 μ*M* and by dissolving thiourea (with sonication) to a concentration of 100 m*M*. 2AT was dissolved in mobile phase and diluted to appropriate final concentrations.

Derivatization reaction

Derivatization of CA was carried out in 75 × 12 mm capped polypropylene tubes containing 10 to 200 μl stock CA solution (2–40 nmol) and 30 μl of stock thiourea (3 μmol) in a final volume of 0.5 ml. The tubes were heated at 90°C for 2 h and cooled to room temperature prior to solid phase extraction. 2-AT constitutes a stable product and reaction mixtures were stored at 8°C for up to 1 week without apparent detriment.

Solid-phase extraction and concentration of 2AT

Solid-phase extraction was performed using Bond Elut SCX cation-exchange columns (100 mg benzenesulfonic acid sorbent, 1 ml column volume; Analytichem, Harbor City, CA, U.S.A.). Columns were prepared by eluting, in order, methanol (1 ml), acetonitrile (1 ml), water (2 × 1 ml) and 1% glacial acetic acid (2 × 1 ml) prior to sample application. All solvents were eluted by gravity and were introduced sequentially when the previous volume level had reached the top of the sorbent; the sorbent was not allowed to dry.

Samples were adjusted to a pH of about 3 with 2 *M* hydrochloric acid (typically 5–6 μl) prior to application of the entire sample volume to a prewashed column. The columns were then washed with 1% acetic acid (4 × 1 ml) followed by 1% acetic acid–methanol (50:50, 3 × 1 ml). Finally, 2-AT was eluted using methanol–14.5 *M* NH_4OH (96:4, 1 ml).

Basic column fractions containing 2-AT were adjusted to a pH of about 6 using approximately 100 μl of 3.2 *M* hydrochloric acid and were concentrated to dryness under a stream of nitrogen with gentle (35°C) warming. The acid serves to convert 2-AT to its hydrochloride salt, eliminating the loss of free 2-AT which is slightly volatile. The residues were reconstituted in 200 μl of mobile phase with vortexing and injected directly.

HPLC

HPLC was performed using a Hitachi Model L-6200 pump, Model L-4000 UV detector and Model D-2000 integrator/recorder. Chromatography was effected on an

Alltech Econosil C_{18} column (250 mm × 4.6 mm I.D.; 10 μm particle size). The mobile phase was acetonitrile–0.05 *M* K_2HPO_4 pH 7 (20:80) which was pumped at a flow-rate of 1 ml/min. Injections were made using a 20-μl Rheodyne loop and the wavelength of detection was 255 nm.

Microsomal incubations

Washed hepatic microsomes from male Sprague-Dawley rats (225–275 g), pretreated with phenobarbital, were prepared according to a published method [9]. Incubation mixtures contained hepatic microsomal protein (0.5 mg), $MgCl_2$ (5 m*M*), CA (2–40 nmol) and 0.04 *M* potassium phosphate buffer, pH 7.4, in a final volume of 0.5 ml and were incubated in open 75 × 12 mm polypropylene tubes for 10 min at 37°C with shaking. Protein was precipitated by addition of, sequentially, 5% $ZnSO_4$ (200 μl) and 2.5% $Ba(OH)_2$ (200 μl). Tubes were centrifuged at 450 *g* for 10 min and supernatant (600 μl) was withdrawn. Stock thiourea solution (30 μl) was added and the derivatization reaction was carried out as described for the samples run in water.

RESULTS AND DISCUSSION

We have developed a novel method to permit quantification of chloroacetaldehyde from microsomal preparations, in which conversion of CA to 2-AT is followed by concentration via a solid-phase cation-exchange column and HPLC. Typical chro-

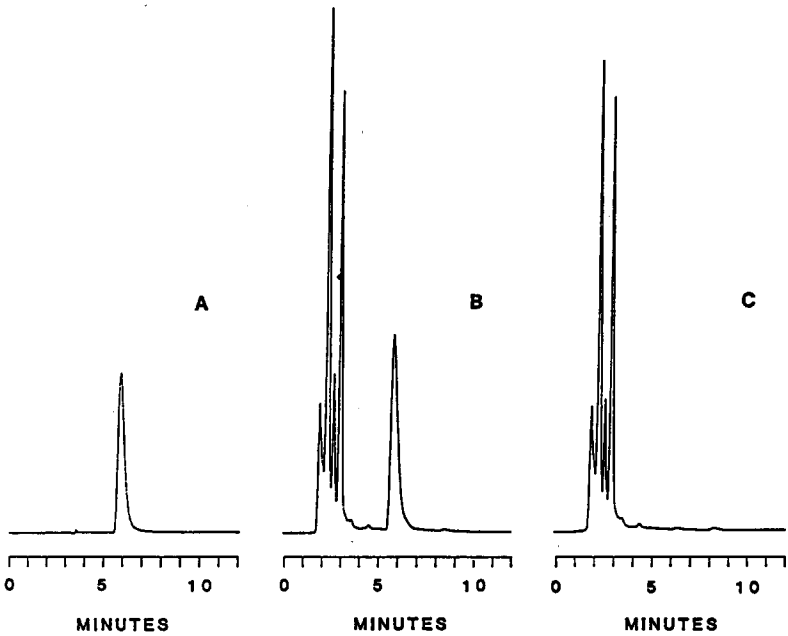

Fig. 2. High-performance liquid chromatograms of (A) authentic 2AT, (B) rat liver microsomal suspension spiked with CA and (C) "blank" rat liver microsomal suspension. Protein-free aliquots (0.5 ml) of B and C, containing, in turn, 16 nmol and 0 nmol of CA, were derivatized with thiourea. Then these and an aliquot of A (5 nmol/0.5 ml) were each subjected to solid-phase extraction. Chromatograms were recorded at 0.01 a.u.f.s. using conditions specified in the Experimental section.

matograms obtained from the work-up of CA spiked RLM incubations are presented in Fig. 2. 2-Aminothiazole exhibited a retention time of 5.9 min and blank test mixtures confirmed the absence of interfering peaks. Chromatograms obtained from aqueous test reactions were virtually identical, revealing the fact that several peaks eluting prior to 2-AT are artifacts of the use of methanol–NH_4OH on the sorbent in SCX columns. These peaks were not eliminated by pre-rinsing SCX columns with methanol–NH_4OH. However, its use was acceptable due to the efficiency of this solvent system in eluting 2-AT, the ease with which it may be concentrated, and the noninterferring nature of the "extra" peaks.

Amounts of 2-AT detected were correlated linearly with amounts of CA initially present. In aqueous samples containing 2-40 nmol of CA, the calibration parameters for the function $y = mx + b$ (where m = nmol/a.u., determined using 6–8 concentrations of CA in 3–4 separate experiments), were as follows: $m = 108 \pm 6$, $b = 0.4 \pm 0.5$ ($r \geqslant 0.998$). In microsomal suspensions analyzed in the same way, $m = 114 \pm 19$, $b = 2.6 \pm 1.1$ ($r \geqslant 0.989$).

The *extent* of derivatization of CA was independent of the *amount* of CA added to aqueous samples (Table I). Yields were calculated from a standard curve constructed from HPLC analysis of 2AT solutions of known concentrations. Comparison of data in Table I indicated that no loss of CA occurred in the presence of liver microsomal protein. The detection limit under these conditions was found to be reached at an initial CA concentration of roughly 2 nmol/0.5 ml. This may be improved marginally by lengthening the heating period of the derivatization, which results in an increase in % conversion of CA to 2-AT (data not shown). This marginal increase was deemed unnecessary for our applications.

CA has been identified as a metabolite of oxazaphosphorine antitumor agents. [1,2,5,10,11]. While its toxicity has not been fully explored, it has been implicated as a source of urotoxicity [10] and neurotoxicity [5] and identified as an alkylating agent [3]. We believe our assay procedure may prove useful to researchers exploring these and other areas in which a straightforward assay of nmol/ml levels of CA may be desired.

TABLE I

CALCULATED YIELDS OF 2-AT FOLLOWING DERIVATIZATION OF CA IN WATER AND IN MICROSOMAL PREPARATIONS

CA concentration (nmol/0.5 ml)	Yield (%, average ± S.E.M.)	
	In water	In microsomal preparation[a]
2.0	22.90 ± 2.42	18.24 ± 5.49
4.0	25.51 ± 1.53	23.01 ± 3.75
8.0	30.26 ± 2.00	27.21 ± 0.36
16.0	26.81 ± 4.28	26.98 ± 2.60
32.0	31.90 ± 1.29	25.26 ± 1.60
40.0	32.30 ± 0.62	25.53 ± 3.41

[a] Yields are adjusted to accomodate the fact that HPLC integration values reflect an aliquot of incubation mixtures removed after protein precipitation.

ACKNOWLEDGEMENT

The authors wish to acknowledge the contribution of Mr. Jay Berman, a participant in the Student Science Training Program of the University of Georgia.

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