Journal of Chromatography, 226 (1981) 514—520
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1030

Note

Reversed-phase high-performance liquid chromatographic assay for the antineoplastic agent 9,10-anthracenedicarboxaldehyde bis(4,5-dihydro-1H-imidazol-2-yl hydrazone) dihydrochloride

GARTH POWIS

Department of Oncology, Mayo Clinic, Rochester, MN 55905 (U.S.A.)

(First received April 22nd, 1981; revised manuscript received July 15th, 1981)

9,10-Anthracenedicarboxaldehyde bis(4,5-dihydro-1H-imidazol-2-yl hydrazone) dihydrochloride (ADAH; (Fig. 1) is an anthracene derivative with antitumor activity against several animal tumor model systems including L-1210 and P-388 murine leukemias, Lieberman plasma cell tumor, B-16 melanoma, Ridgeway osteogenic sarcoma and murine colon tumor 26 [1]. Studies in dogs show that ADAH exhibits less cardiotoxicity than adriamycin [2]. ADAH is currently undergoing clinical evaluation as an anticancer agent in humans. There is no information on the pharmacologic behavior of ADAH in humans. As a preliminary step to studies in humans a sensitive reversed-phase high-performance liquid chromatographic (HPLC) assay for ADAH in plasma and other biological fluids has been developed. The assay has been used to study the disposition of ADAH in rabbit and a patient receiving ADAH in phase II clinical trial.

EXPERIMENTAL

Drugs

9,10-Anthracenedicarboxaldehyde bis(4,5-dihydro-1H-imidazol-2-yl hydrazone) dihydrochloride (CL 216,942) was supplied by Dr. K.C. Murdock, American Cyanamid Company, Lederle Laboratories, Pearl River, NY, U.S.A.; 1-[2-(2-hydroxyethyl-amino)ethylamino]-4-hydroxy-9,10-anthracenedione (NSC 299,187) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A.; β-glucuronidase was obtained from Boehringer Mannheim, Indianapolis, IN, U.S.A.

0378-4347/81/0000—0000/$02.50

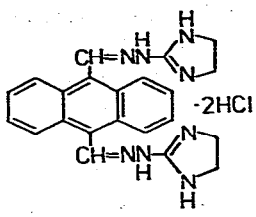

Fig. 1. Structure of ADAH.

Animal and patient studies

Male New Zealand white rabbits weighing between 2 and 3 kg were injected intravenously with ADAH in 3 ml 0.9% NaCl containing 10% (w/v) ethanol at doses between 50 and 150 mg/m^2, over a 1-min period into a peripheral ear vein using a vein infusion set with a winged adapter (Miniset, Travenol, Deerfield, IL, U.S.A.). Blood was collected into heparinized tubes at different times from a peripheral vein of the other ear vein. Urine was collected for 24 h following administration of the drug by placing the animal in a stainless-steel metabolism cage. The bile duct of one rabbit was cannulated and the bladder catheterized through the urethra under pentobarbital anesthesia. The animal was allowed to recover for 3 h before being given ADAH, and bile and urine were collected from the exteriorized bile cannula and bladder catheter for 24 h. ADAH was administered to a patient with cancer at a dose of 260 mg/m^2 as a 90-min intravenous infusion as part of a phase II clinical trial.

Preparation of samples

A 2-ml volume of plasma, or a lesser volume of urine or bile diluted to 1 ml with water, was mixed with an equal volume of 1 *M* sodium phosphate buffer (pH 10) and extracted with 8 ml of ethyl acetate. The efficiency of extraction of ADAH was 95%. In most cases a standard of 1 μg of 1-[2-(2-hydroxyethyl-1-amino)ethylamino] 4-hydroxy-9,10-anthracenedione (alkyl AAD) was added to the sample prior to extraction to provide an internal measure of extraction efficiency. The ethyl acetate extract was taken to dryness under nitrogen and the residue dissolved with gentle warming in 300 μl of methanol. A 100-μl sample was taken for HPLC. Bile and in some cases urine samples, 0.5 ml, were mixed with 50 μl of 1 *M* sodium acetate buffer (pH 4.0) and incubated overnight with β-glucuronidase, 10 U/ml, prior to extraction.

High-performance liquid chromatography

ADAH was separated by reversed-phase HPLC on a 25-cm C_2-bonded LiChrosorb RP-2 column, 5 μm particle size (E. Merck, Darmstadt, G.F.R.) with a 10-min linear gradient of 5% to 100% methanol in 0.5 *M* sodium perchlorate (pH 5.3) at a flow-rate of 2 ml/min. The sodium perchlorate solution was cleaned by passing it through a bed of silica gel and activated charcoal before use. Eluting compounds were detected at 430 nm. Alkyl AAD was measured by a second chromatographic run with detection at 500 nm. A Hewlett-Packard 1084B liquid chromatograph and variable-wavelength detector

798575A were employed. Reproducibility of injection was < 1%. Wavelengths are uncorrected values. The output from the detector was fed into a Hewlett-Packard 79850B liquid chromatograph terminal and peak areas integrated.

Pharmacokinetic analysis

Nonlinear least-square regression analysis of the data to obtain pharmacokinetic parameters [3] employed the SAS NLIN pharmacokinetic program with a weighting factor of $1/y^2$ [4]. Allowance was made for a 1-min infusion of drug in rabbit.

RESULTS AND DISCUSSION

ADAH was bound tightly to the C_2-bonded reversed-phase support and could not be eluted with acetonitrile as the mobile phase although it was eluted as a broad peak with methanol. When a gradient of 5% to 100% methanol in 10 m*M* sodium acetate (pH 4.0) buffer was used ADAH still eluted as a broad peak. A gradient of 5% to 100% methanol in 0.1 *M* to 0.5 *M* sodium perchlorate at acid pH produced a sharp ADAH peak. Optimum resolution was obtained by a linear gradient of 5% to 100% methanol in 0.5 *M* sodium perchlorate (pH 5.3). Sodium perchlorate contained contaminants which bound to the column and eluted with methanol, interfering with the assay for ADAH. These contaminants were removed by passing the sodium perchlorate solution through a bed of silica gel and activated charcoal before use.

Freshly prepared ADAH dissolved in methanol or plasma eluted as a single peak on HPLC (Fig. 2). The lower level for detection of ADAH in rabbit plasma was 25 ng/ml and the assay was linear up to least 10 μg/ml. Urine and bile samples were diluted appropriately to bring ADAH levels into this range. The coefficient of variation of the assay at 0.1 μg of ADAH per ml of plasma was ± 7.3%. Alkyl AAD was used as an internal standard for the extraction procedure. We were unable to find a suitable internal standard which

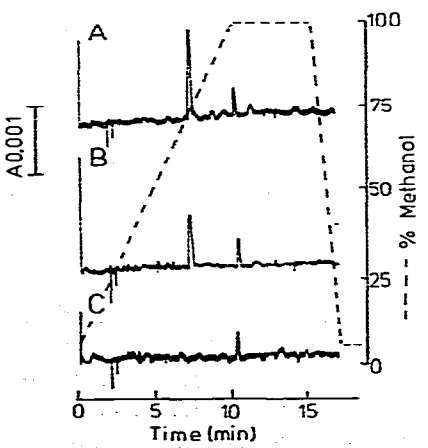

Fig. 2. Chromatograms of ADAH added to rabbit plasma: (A) 50 ng/ml; (B) 25 ng/ml; and (C) control plasma. Chromatographic conditions are described in the text. Detection at 430 nm.

could be measured at the same detector wavelength as ADAH. Alkyl AAD had therefore to be determined separately in a second chromatographic run with a detector wavelength of 500 nm.

If a sample of 10 μg of ADAH in methanol was exposed to fluorescent room light for 12 h, additional peaks were formed (Fig. 3). There was a major peak eluting before the peak due to ADAH and a smaller peak eluting after the peak due to ADAH. The formation of additional peaks also occurred with nitrogen-saturated ADAH solution exposed to light. Equilibrium between the forms represented by the different peaks appeared to have been reached by 12 h and exposure to light for periods of up to 40 h produced no further change in the pattern of the peaks. The absorption spectra of the new major peak, of parent ADAH and of the third smaller peak were similar with maxima at, respectively, 270 nm, 375 nm and 432 nm (uncorrected wavelengths, Hewlett-Packard variable-wavelength detector).

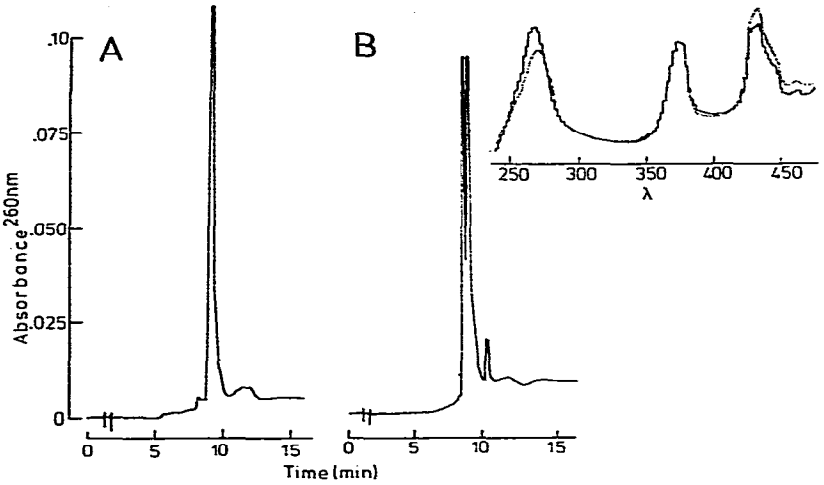

Fig. 3. Chromatograms of (A) 1 μg of ADAH prepared as a 10 μg/ml solution in methanol and stored overnight at room temperature in the dark, and (B) 1 μg of ADAH, prepared as a 10 μg/ml solution in methanol and placed on a bench surface under fluorescent room lights overnight. Inset are wavelength scans of the two major peaks, full line the first peak which eluted, dotted line the second peak which eluted (which had the same retention time as parent ADAH). Chromatographic conditions are described in the text.

ADAH is administered to humans by infusion dissolved in 5% (w/v) dextrose at a concentration of approximately 1 mg/ml. There was no detectable conversion of ADAH to other components when 1 mg of ADAH per ml of 5% dextrose was exposed to light for 4 h. However, a more dilute solution, 10 μg of ADAH per ml of 5% dextrose, showed a 13.6% conversion to a more rapidly eluting peak when exposed to light for 4 h. The nature of the additional peaks is not known. One possibility is that the peaks represent the three possible isomers of ADAH resulting from the *syn* and *anti* configurations of the hydrazone groups. When assaying ADAH in plasma, urine and bile, a second peak eluting before the parent ADAH peak was always apparent despite precautions to keep the samples dark. Consequently, in the assay for ADAH

both peaks were added together. By limiting exposure of the samples to light the peak eluting before parent ADAH was usually less than 10% of the total area.

Plasma concentrations of ADAH in the rabbit fell rapidly following intravenous injection of ADAH (Fig. 4). The data were best described by a two-compartment open model. Pharmacokinetic parameters are shown in Table I. Relatively little unchanged ADAH was excreted in the urine. The 24-h urinary excretion of ADAH at doses of 50, 100 and 150 mg/m^2 was 11.7, 5.8 and 7.4% of the dose administered, respectively. In 24 h 7.8% of ADAH administered was excreted unchanged in the bile of an animal with a cannulated bile duct receiving 100 mg of ADAH per m^2 (Fig. 5). A small amount of ADAH was excreted in the bile as a glucuronide conjugate detected by an increase in free ADAH after β-glucuronidase digestion. In the rabbit in which bile samples were collected, 2.6% of the administered dose of ADAH was excreted in the urine in 24 h as unchanged ADAH and 3.8% as a glucuronide conjugate of ADAH.

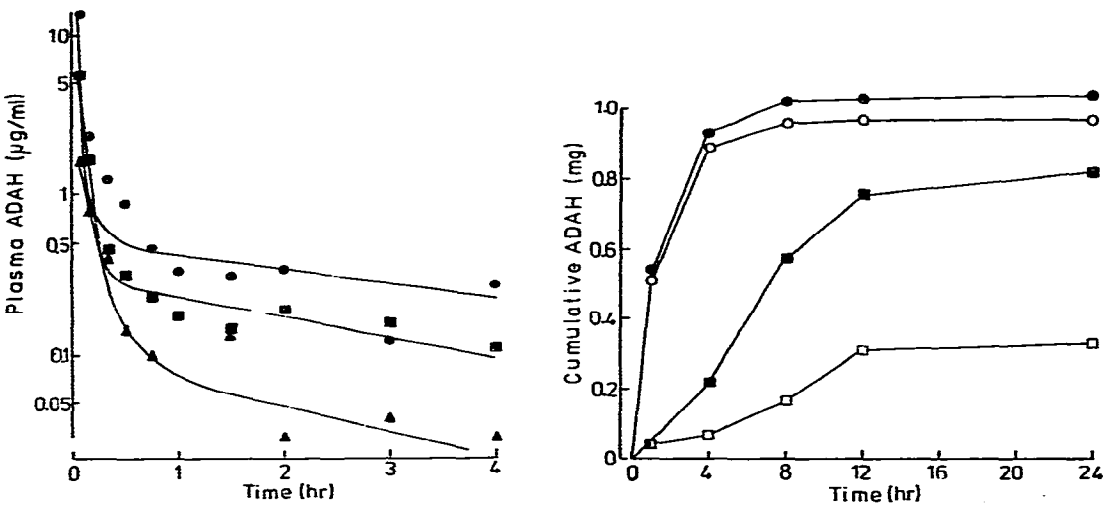

Fig. 4. Plasma ADAH concentration in rabbit plasma following intravenous injection of ADAH: (▲) 50 mg/m^2, (■) 100 mg/m^2, and (●) 150 mg/m^2. Points are experimental observations, continuous lines are computer fits to the data.

Fig. 5. Biliary (●,○) and urinary (■,□) excretion of ADAH. The rabbit received 12.5 mg of ADAH (100 mg/m^2). Bile and urine were collected at various times for 24 h through an exteriorized bile cannula and bladder catheter. The filled symbols represent total ADAH after treatment of the sample with β-glucuronidase, the open symbols free ADAH without β-glucuronidase treatment.

Control human plasma contained peaks detectable at 430 nm not seen in rabbit plasma. The lower limit for detection of ADAH in human plasma was 50 ng/ml. Chromatograms of patient plasma before and after receiving ADAH are shown in Fig. 6. Plasma concentrations of ADAH in the patient fell relatively rapidly following cessation of the intravenous infusion of ADAH but ADAH could still be detected 24 h later (Fig. 7). The concentration of ADAH in

TABLE I

PHARMACOKINETIC PARAMETERS OF ADAH IN RABBIT

Rabbits were administered ADAH at the doses shown. $t_{½\alpha}$ and $t_{½\beta}$ are the distributive and post-distributive half-lives. V_1 and V_2 the volumes of the central and peripheral compartments, and Cl the total body clearance of ADAH.

Dose (mg/m²)	$t_{½\alpha}$ (min)	$t_{½\beta}$ (min)	V_1 (l/m²)	V_2 (l/m²)	Cl (l/min/m²)
50	5.7	113.0	17.6	495.6	1.30
100	1.8	138.3	2.3	313.8	0.57
150	1.9	201.2	1.5	288.2	0.36

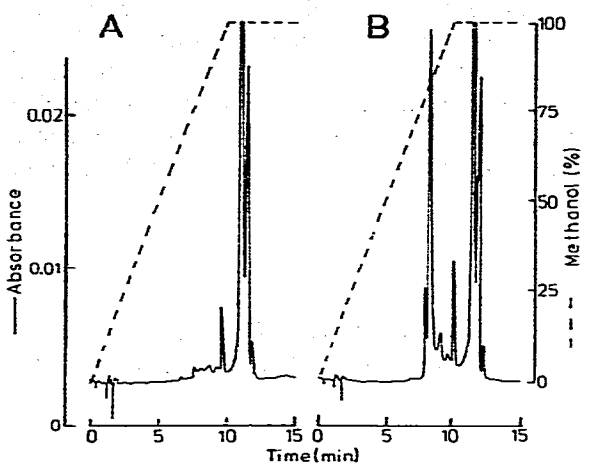

Fig. 6. Chromatograms of patient plasma: (A) before treatment; (B) 60 min after receiving a 90-min infusion of ADAH, 260 mg/m². This sample contained 1.0 μg of ADAH per ml.

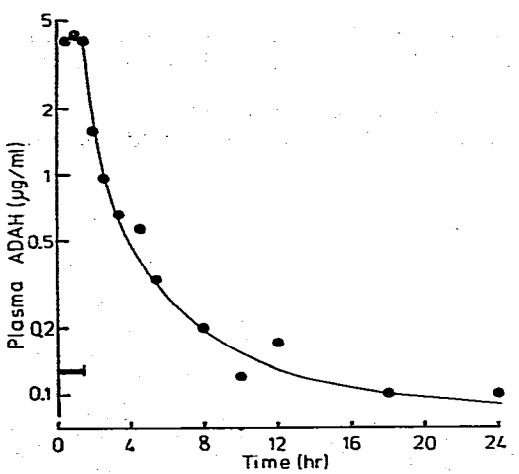

Fig. 7. Plasma ADAH concentration in a 62-kg female cancer patient receiving intravenous infusion of ADAH, 260 mg/m² over 90 min (shown by the bar).

plasma 24 h after administration was above the level, 50 ng/ml for 1 h, that completely inhibits growth of some human tumor cell lines in the in vitro soft agar colony forming assay [5].

In summary, a sensitive reversed-phase HPLC assay for ADAH has been developed which is suitable for measuring ADAH in the plasma and urine of humans receiving the drug in clinical trial. ADAH is sensitive to light and in solution exposed to light forms a second and a third component which can be separated from the parent ADAH by HPLC. The three peaks have similar absorption spectra. Studies in the rabbit have shown that plasma ADAH concentrations fall rapidly in a biphasic manner following intravenous administration of the drug, $t_{½\alpha}$ = 3.1 min and $t_{½\beta}$ = 151 min and a mean total body clearance of 743 ml/min/m^2. Between 6 and 12% of the dose of ADAH is excreted unchanged in the urine in 24 h. A further small proportion of ADAH is excreted in the urine as glucuronide conjugates. In a rabbit with a cannulated bile duct 8% of a dose of ADAH was excreted unchanged in the bile in 24 h, mostly in the first 4 h after ADAH administration. Preliminary studies in a patient receiving ADAH have shown that plasma ADAH concentrations also fall rapidly but that ADAH can still be detected in the plasma 24 h later. Plasma concentrations of ADAH have been measured in a patient receiving ADAH in Phase II clinical trial.

ACKNOWLEDGEMENTS

The helpful discussions of Dr. Mark Sanders are gratefully acknowledged. This work was supported in part by NCI Contract CM 97273 and by American Cancer Society Grant CH 143.

REFERENCES

1 R.V. Citarella, R.E. Wallace, K.C. Murdock, R.B. Angier and F.E. Durr, 20th Intersc. Conf. Antimicrob. Agents Chemother., American Society of Microbiology, Washington, DC, 1980, Abstr. No. 23.
2 B.M. Sparano, G. Gordon, C. Hall, M.J. Iatropoulos and T.F. Noble, 20th Intersc. Conf. Antimicrob. Agents Chemother., American Society of Microbiology, Washington, D.C, 1980, Abstr. No. 31.
3 M. Gibaldi and D. Perrier, Pharmacokinetics, Marcel Dekker, New York, 1975.
4 A.J. Barr, J.H. Goodnight, J.P. Sall and J.T. Helwig, A User's Guide to SAS 76, SAS Institute, Sparks Press, NC, 1976.
5 M. Lieber and G. Powis, unpublished results.