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DETERMINATION OF L-ALANOSINE IN PLASMA AND URINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE Dns DERIVATIVE

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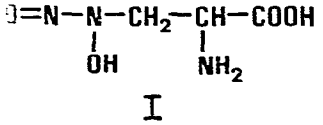
SUMMARY

L-Alanosine is an antitumour antibiotic that has recently been placed in clinical trial. We have developed a relatively rapid and specific assay for urinary and plasma alanosine, based on formation of the Dns derivative and separation of this from other Dns compounds by reversed-phase high-performance liquid chromatography. Dns-Alanosine is detected by its absorption at 254 nm, since alanosine is atypical in that it forms a Dns derivative with very low fluorescence. The lower limit of detection of alanosine in plasma is 0.1 μg/ml. The assay has been used to measure the levels of alanosine in the plasma and urine of rabbits and of man.

INTRODUCTION

Alanosine is an antibiotic produced by *Streptomyces alanosinicus*; it has antiviral and imminosuppressive properties[1–3], and exhibits marked activity against several experimental tumours in mice[4,5]. The structure of the compound has been shown to be L-(−)-2-amino-3-(hydroxynitrosamino)propionic acid (I)[2]. Initial studies in bacteria showed that alanosine depresses AMP and pyrimidine biosyntheses, and it was suggested that it was an inhibitor of adenylosuccinate synthetase and aspartate transcarbamylase[6]. More recent studies with Novikoff hepatoma cells have shown that alanosine specifically inhibits adenylosuccinate synthetase and has no effect on pyrimidine biosynthesis[7]. Alanosine itself does not inhibit adenylosuccinate synthetase[8,9], and a metabolite (probably the alanosine analogue of N-succinylcarboxamide-amino imidazole ribotide) has been suggested as the inhibitor of the enzyme[10]. L-Alanosine has been placed in clinical trial as an antitumour agent, and we wished to be able to



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measure the levels of the drug in the plasma and urine of patients receiving the compound. No assay for alanosine, apart from determination of total radioactivity, has been published in the literature. The ability to measure the levels of circulating alanosine is particularly important, since studies *in vitro* have suggested that the growth-inhibitory effects of alanosine are reversed once the drug is removed[7]. We report here a convenient and specific method for determining alanosine, based on separation of the Dns derivative of alanosine from other compounds in plasma and urine by gradient high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Preparation of Dns derivatives

Plasma. Plasma was adjusted to pH 8.0 with 1 *N* NaOH, and 1 ml was mixed with 2 ml of 5 m*M* Dns-Cl (dansyl chloride; 5-dimethylaminonaphthalene-1-sulphonyl chloride; Eastman Kodak, Rochester, N.Y., U.S.A.) in acetone and incubated at 37° for 1 h. Complete precipitation of the plasma proteins was achieved by keeping the mixture at −20° for a further 1 h and centrifuging at 10,000 *g* for 10 min at −5°. A 2-ml portion of the supernatant liquid was removed, frozen in a bath of solid CO_2 and acetone and lyophilised; the residue was dissolved in 0.1 ml of 50% aqueous acetone, and 10 µl were injected into the HPLC system.

Urine. The urine was adjusted to pH 7.0 with either 1 *N* HCl or 1 *N* NaOH and applied to a column containing 1 ml of anion-exchange resin (AG 1-X8; 100–200 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.), equilibrated with 0.1 *M* K_2HPO_4. The column was washed twice with 0.5 ml of water, and bound amino acids and alanosine were then eluted with four × 0.5-ml portions of 0.1 *M* sodium phosphate buffer (pH 9.0) in 2 *N* NaCl. The combined eluate was mixed with 3 ml of 5 m*M* Dns-Cl in acetone and incubated at 37° for 1 h. The mixture was then frozen and lyophilised. The residue was shaken with 3 ml of ethyl acetate for 10 min and centrifuged for 10 min at 1000 *g*. A 2-ml portion of the supernatant liquid was removed and dried under nitrogen, and the residue was dissolved in 0.1 ml of 50% aqueous acetone ; 10 µl were injected into the HPLC system.

High-performance liquid chromatography

Dns-Alanosine was separated from other Dns compounds by reversed phase HPLC on a 25-cm C_8-bonded LiChrosorb RP-8 column (Merck, Darmstadt, G.F.R.) with a gradient of acetonitrile (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.) in 0.01 *N* sodium acetate buffer (pH 4.0) as mobile phase (flow-rate 2 ml/min); the eluting compound was detected at 254 nm, a Hewlett-Packard 1084B liquid chromatograph and variable-wavelength UV detector were employed. The output from the detector was fed into a Hewlett-Packard 79850B liquid chromatograph terminal, and peak areas were integrated. Fluorescence measurements of reference compounds were made on an SLM Series 8000 spectrofluorimeter.

RESULTS AND DISCUSSION

For the best sensitivity, Dns-amino acids are normally detected by their fluorescence emission[11]. The Dns derivative of alanosine differs from other Dns-amino

acids in its fluorescence spectrum and intensity of fluorescence. When dissolved in 37% acetonitrile in 0.01 *N* sodium acetate buffer pH 4.0 (the medium in which it is eluted from the column), Dns-alanosine, excited at 340 nm, has an emission maximum at 475 nm, compared with 515 nm for Dns-lysine. The fluorescence emission maxima for Dns-alanosine and Dns-lysine in ethanol are 455 and 502 nm, respectively, and the quantum yield of Dns-alanosine is only 17% of that of Dns-lysine. We suspect that alanosine is probably dansylated at both the primary-amine and the N-hydroxyl groups. It has been observed that O,N-bis-Dns-*p*-tyramine, although having two apparently independent fluorophores, exhibits a much lower fluorescence efficiency than the analogous amine derivative[12,13]. As, in neither instance would the two fluorophores be connected by a system of conjugated double bonds, an intramolecular interaction of the π-electron systems of the two fluorophores might be assumed.

A typical chromatogram of human plasma to which L-alanosine had been added is shown in the upper panel of Fig. 1. The optimum pH for forming the Dns derivative of alanosine was 8.0. Removal of plasma proteins by acetone precipitation before addition of Dns-Cl resulted in the loss of 75% of the alanosine. The maximum yield of Dns-alanosine in plasma was 60% of that of an alanosine standard in buffer solution of pH 8.0. This was constant, and reproducible standard curves such as that

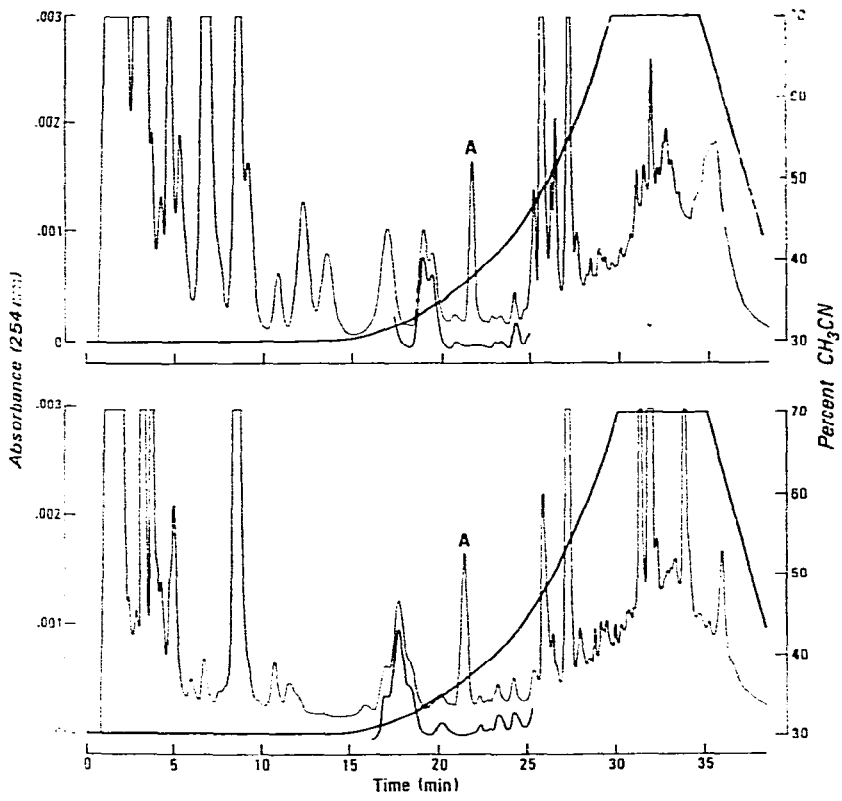

Fig. 1. Chromatograms of human plasma (upper panel) and human urine (lower panel) to which has been added L-alanosine (15 μg/ml). Derivative formation and HPLC were as described in the text. A = alanosine. The insert in each panel is part of a chromatogram of a sample containing no alanosine. The curve is the gradient [percentage of acetonitrile in 0.01 *N* sodium acetate buffer (pH 4.0)].

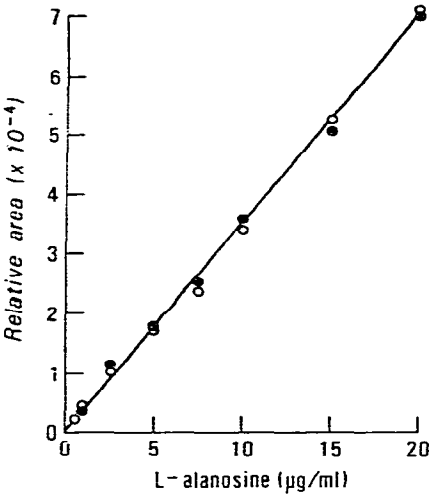

Fig. 2. Standard curves for L-alanosine added to plasma (○) and urine (●).

shown in Fig. 2 could be obtained. The limit of detection of alanosine in plasma was 0.1 μg/ml.

Urine contained many compounds (not detected in plasma) that formed Dns derivatives, and it was necessary partially to purify the alanosine by anion-exchange chromatography before Dns derivatization; this procedure removed most of the interfering compounds. A typical chromatogram of human urine to which L-alanosine had been added is shown in the lower panel of Fig. 1, and a standard curve for alanosine in urine is shown in Fig. 2. The detection limit of alanosine in urine was 0.5 μg/ml, somewhat higher than in plasma. Sensitivity in the analysis of urine was not an important factor in our studies, since the levels of alanosine in urine were well above the limit of detection.

Derivatives of Dns-Cl have been used widely for the separation and fluorimetric detection of amino acids and for end-group determination of proteins and peptides[12]. Recently, Dns-amino acids have been separated by HPLC on ion-exchange resin[14], polyamide[15], and silica[16]. None of these methods has been applied to amino acids in biological fluids, and they all require considerable time for each assay. With the method described here, the choice of an appropriate elution gradient allows efficient separation of Dns-alanosine from other Dns derivatives, including those of amino acids, within a relatively short time.

The plasma levels and cumulative urinary excretion of alanosine administered intravenously over 20 sec to rabbits at doses of 150 or 500 mg/m² are shown in Fig. 3. Alanosine is rapidly cleared from the plasma with a decrease in the plasma concentration of more than 100-fold in 2 h. At the higher dose level, the elimination phase of alanosine in the plasma had a half-life of 83 min, and the initial distributive phase a half-life of 5 min. The 24-h urinary excretion of alanosine was 6 and 16% of the dose injected, at the low and high dose levels, respectively, and most of the alanosine in the urine was excreted within 90 min of administration. The doses of L-alanosine administered to the rabbits were chosen as those which might be expected to encompass those used in man. L-Alanosine is currently being administered to patients in Phase I

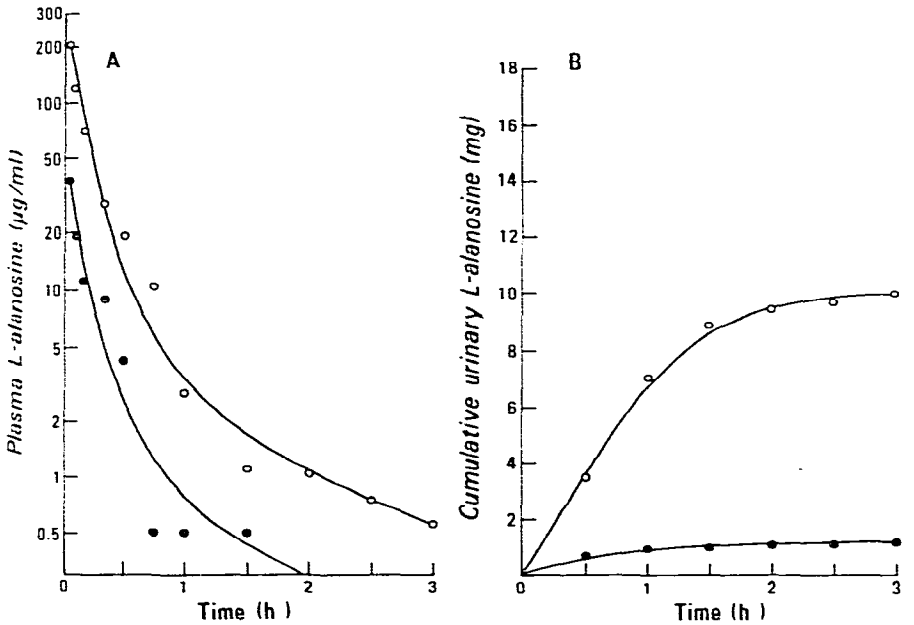

Fig. 3. Plasma levels (A) and cumulative urinary excretion (B) of alanosine following intravenous administration of L-alanosine to male rabbits at a dose of 150 mg/m² (●) and 500 mg/m² (○).

clinical trial at a dose of 250 mg/m². Plasma levels of alanosine administered to one patient at a dose of 250 mg/m² over a period of 10 min are shown in Fig. 4. As in the rabbit, alanosine is rapidly cleared from the plasma; the half-life of the final elimination phase was 159 min.

Studies on the levels of total radioactivity in the plasma after the administration of DL-[1-^{14}C]alanosine to mice, rats, dogs and monkeys have been reported, but only in one study (in the mouse) was an attempt made to distinguish between DL-alanosine and its metabolites[17]. Metabolites accounted for more than 85% of the total radioactivity only a few minutes after intravenous administration. The half-lives for the rapid and slower phases of removal in the mouse can be calculated from the data of Kelley *et al.*[17] to be 10 and 156 min, respectively. The elimination phase for DL-alanosine in mice thus appears to be longer than that for L-alanosine in the rabbit, but comparable to that in man.

The 24-h urinary excretion of alanosine in man was 5.6% of the dose injected, with 40% of this excreted in the 4 h following administration. The 24-h urinary excretion of total radioactivity following the administration of DL-[1-^{14}C]alanosine to a variety of experimental animals is 40–60%[17]. It is therefore probable that, in man and rabbit, urinary metabolites will account for most of the alanosine-derived compounds excreted.

Following the administration of the large dose of L-alanosine to the rabbit, there appeared on the chromatogram for plasma a peak with a retention time of 6 min under the conditions described; a similar peak was observed in plasma from man. This peak has yet to be identified, and it could be an amino acid or a metabolite of alanosine.

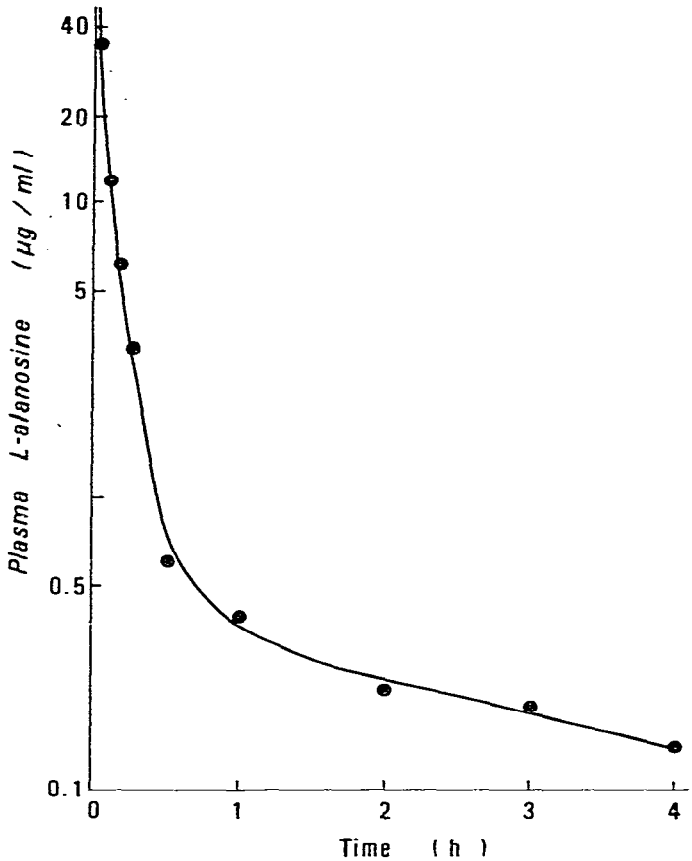

Fig. 4. Plasma levels of alanosine in a female patient receiving L-alanosine at a dose of 250 mg m^{-2} as an intravenous infusion over 12 min; zero time is taken as being the end of the infusion.

The reversed-phase HPLC system we have described can be used, with appropriately modified gradients, to detect changes in the levels of other amino acids in the plasma and to determine their rate of urinary excretion. This may be of value, since alanosine has been reported to be a dicarboxylic acid antagonist, producing glutamic aciduria and aspartic aciduria in experimental animals[18]; measurement of such changes might be used to assess the biological activity of the drug in man. By lowering the initial concentration of acetonitrile in the gradient, more rapidly eluting components, including Dns-aspartic acid and Dns-glutamic acid, can be resolved.

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