Journal of Chromatography, 181 (1980) 95—99
Biomedical Applications

CHROMBIO. 427

Note

Determination of 6-diazo-5-oxo-L-norleucine in plasma and urine by reversed-phase high-performance liquid chromatography of the dansyl derivative

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(Received June 11th, 1979)

6-Diazo-5-oxo-L-norleucine (DON) is a glutamine antagonist and a potent inhibitor of L-asparagine synthetase [1, 2] and several L-glutamine amidotransferases concerned with purine biosynthesis [2, 3]. It has antitumor activity in a variety animal tumor system [4, 5]. Although DON has been used clinically [1, 4, 6], there has been no comprehensive study of the drug in man. Interest in DON has revived because of the effectiveness of the closely related azotomycin in certain types of cancer [7, 8] and because of the hope that inhibitors of L-asparagine synthetase might prevent the development of clinical resistance to therapy with L-asparaginase which has been linked to an increase in L-asparagine synthetase activity [9]. DON is currently being studied in Phase I clinical trials.

Microbiological procedures have been reported for the measurement of DON in plasma and urine [5, 6]. These microbiological assays are sensitive but lack specificity and several endogenous intermediary metabolites have been shown to affect the degree of inhibition of microbial growth produced by DON [5]. We report a specific and sensitive assay for DON based upon the separation of the dansyl derivative of DON from other dansyl derivatives in plasma and urine by reversed-phase high-performance liquid chromatography (HPLC).

EXPERIMENTAL

One ml of rabbit plasma containing DON was mixed with 2 ml of 5 m*M* dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride; Eastman Kodak, Rochester, N.Y., U.S.A.) in acetone. The mixture was incubated at 37° for 60 min in closed tubes and left at −20° for 60 min. Precipitated proteins were removed by centrifuging at 10,000 *g* for 10 min at −10°. Two ml of the

supernatant fluid were removed, frozen in a dry ice—acetone bath and lyophilized. The residue was dissolved in 0.2 ml of 50% acetone in water and 20 μl taken for chromatography. Urine was treated in a similar manner except that the pH was first adjusted to 9.0. It was not necessary to adjust the pH of plasma. Reversed-phase chromatography was carried out on a C_8-bonded Zorbax RP-8 column (4.5 × 250 mm) (DuPont, Wilmington, Del., U.S.A.) with 10% acetonitrile (Burdick & Jackson Labs., Muskegon Mich., U.S.A.) in 0.01 *N* sodium acetate buffer, pH 7.2 with a flow-rate of 1.7 ml/min and a column temperature of 55°. A Hewlett-Packard 1084B microprocessor-controlled high-performance liquid chromatograph and variable wavelength UV detector were employed. Eluting peaks were detected by their absorbance at 254 nm and peak areas integrated on a Hewlett-Packard 79850B liquid chromatograph terminal.

A logarithmic-ratio microbiological assay for DON using *Escherichia coli*/ACB-C (supplied by Dr. W.J. Suling, Southern Research Institute, Birmingham, Ala., U.S.A.) as described by Cooney et al. [5] was employed for purposes of comparison.

DON was administered to male New Zealand white rabbits, weighting 2.5 to 3 kg, into a marginal ear vein over 30 sec. Blood was collected into heparinized tubes at various intervals from the other ear vein. Urine was collected from a catheter inserted into the bladder under light ketamine (Ketaject, Bristol Laboratories, Syracuse, N.Y., U.S.A.) anesthesia one hour prior to the study. The bladder was flushed at each collection with 5 ml sterile saline. Rabbits were placed in a metabolism cage for the collection of 24-h urine. Pharmacokinetic analysis of plasma drug levels was conducted using the SAS NLIN non-linear least squares regression analysis program [10]. DON (NSC-7365) was supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., U.S.A., and stored desiccated at −20°.

RESULTS AND DISCUSSION

The conditions of dansylation described in Experimental were determined to be optimum for the formation of the dansyl derivative of DON in plasma and urine. The dansyl derivative of DON was completely resolved from other dansyl derivatives in plasma as a peak eluting at approximately 30 min (Fig. 1). Dansyl chloride and less polar dansyl derivatives were removed from the column by gradually increasing the acetonitrile in the solvent mixture to 100% over 12 min. This procedure was necessary for consistent column performance. The assay could detect 1.0 μg DON per ml. A representative standard curve for DON added to plasma is shown in Fig. 2. Dansyl DON can also be detected fluorometrically with an excitation wavelength of 340 nm. DON was found to be relatively unstable in solution and all biological samples were frozen after collection and assayed within 24 h.

Plasma decay curves for DON following administration to rabbits at doses of 400 and 600 mg/m^2 (23 and 34 mg/kg) are shown in Fig. 3 (the anticipated starting dose for the clinical study is 300 mg/m^2). The HPLC assay gave slightly lower values for plasma DON at later time points but otherwise the two assays gave similar plasma decay curves. The lower limit of detection of DON in rabbit

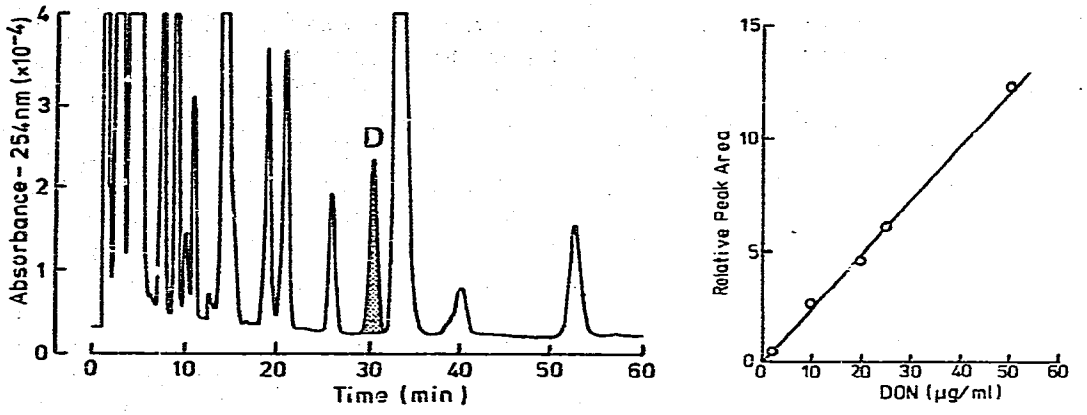

Fig. 1. Chromatogram of rabbit plasma to which DON had been added to give a concentration of 25 µg/ml. The plasma was derivatized and chromatographed by reversed-phase HPLC as described in the text. The peak formed by DON is shaded and identified by D. The baseline under the peak is the chromatographic pattern of the same plasma not containing DON.

Fig. 2. Standard curve for DON in plasma determined by reversed-phase HPLC.

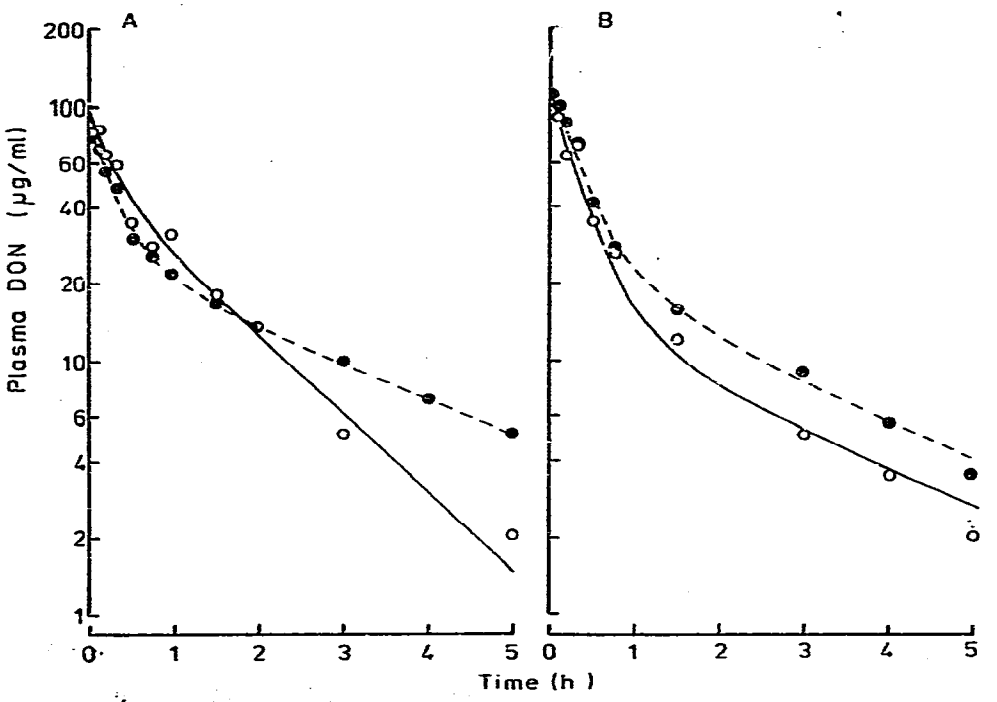

Fig. 3. Plasma levels of DON following the intravenous administration to rabbits of DON at doses of (A) 400 mg/m² (23 mg/kg) and (B) 600 mg/m² (34 mg/kg). ○, Determined by reversed-phase HPLC; ●, determined by the microbiological procedure. The lines are the computer plots of the data: —, for the HPLC data; — — —, for the microbiological data.

and human plasma by the microbiological procedure was 1.0 μg/ml, a sensitivity comparable to the HPLC assay. A ring of dense bacterial overgrowth immediately adjacent to the disk to which the plasma was applied limited the sensitivity of the assay. This overgrowth did not occur with mouse plasma which was used for the development of the original assay [5]. The removal of DON from the plasma measured by the HPLC assay was biphasic with mean pharmacokinetic parameters of $t_{1/2}^{\alpha}$, 11.1 min; $t_{1/2}^{\beta}$ 85.3 min; V_D^{α}, 284 ml/kg; V_D^{β}, 880 ml/kg and a clearance of 5.3 ml/min/kg body weight. No DON could be detected in the urine with either the chemical or microbiological assays.

Cooney et al. [5] using a microbiological assay, reported a monoexponential decline in plasma DON with an apparent half-life of ca. 30 min, following intraperitoneal administration of DON to mice. Studies by Magill et al [6], using a microbiological assay, showed biexponential plasma decay with a rapid distributive phase and a slower post-distributive phase with a half-life of 1—2 h in patients given DON intravenously. In the present study the agreement between the plasma concentration of DON as determined by the HPLC assay specific for DON and the microbiological assay suggest that in the rabbit, metabolites with growth inhibitory properties are not present in large amounts in plasma or urine. A slowly eluting peak (elution time ca. 52 min) was detected by the HPLC assay. The area of this peak, which has yet to be identified, increased with time.

Separation of dansyl amino acids by HPLC has been reported on ion-exchange resin [11], polyamide [12], silica [13], and more recently reversed-phase media [14]. None of these methods has been applied to amino acids in biological fluids. With the method we have developed, dansyl DON can be separated under isocratic conditons from other dansyl derivatives including amino acids within relatively short periods of time.

ACKNOWLEDGEMENTS

The excellent technical assistance of Ms. Margo Sternad is gratefully acknowledged. The assay method is based upon a procedure for the reversed-phase separation of dansyl amino acids developed by Jacque Elion and Kenneth Mann (unpublished work). The study was supported in part by Grant CM 97273A and Grant 15083 F.F., N.C.I., N.I.H., and a grant from the National Eagles Cancer Fund.

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