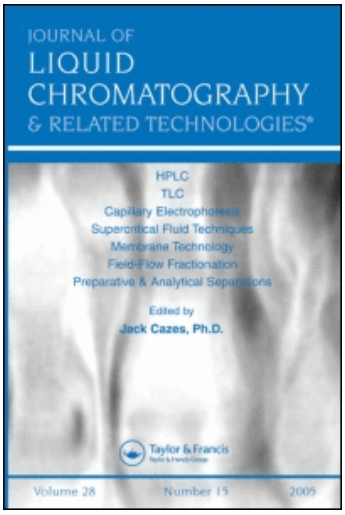

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RAPID ANALYSIS OF 6-DIAZO-5-OXO-L-NORLEUCINE (DON) IN HUMAN PLASMA AND URINE

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ABSTRACT

A paired-ion, reversed phase high pressure liquid chromatography (HPLC) procedure is described for the analysis of DON in human plasma and urine. Plasma proteins are removed by centrifugal membrane filtration, and the filtrate is injected directly onto an octadecylsilane column. The DON is eluted in a mobile phase consisting of 5 mM 1-heptanesulfonic acid, pH 2.4. Eluting material is monitored at 280 nm and 254 nm. The lower limit of sensitivity in plasma is 0.1 μg/ml.

INTRODUCTION

Renewed interest in the glutamine antagonist, 6-diazo-5-oxo-L-norleucine (NSC-7365; DON), has resulted from observations of its chemotherapeutic activity against human tumor lines implanted into nude mice and its activity in human clinical trials (1,2). The new clinical trials of this agent employ an intermittent, relatively high intravenous dose (2) whereas many of the

earlier trials utilized an oral preparation of the drug given on a chronic schedule (3). DON presents a problem to the analytical chemist since it is relatively unstable at extremes of temperature and pH (4). Furthermore, it is an amino acid analog and many methods for its selective extraction and analysis are complicated by the high levels of chemically-similar substances present in biological fluids. Fortunately, the compound has appreciable ultraviolet absorbance at 274 nm (reported $E_{1cm}^{1\%}$ =683, reference 4). We have taken advantage of this property of DON in developing the paired-ion, reversed phase HPLC method of analysis reported herein.

MATERIALS AND METHODS

The bulk chemical and pharmaceutical preparations of DON were supplied by the Pharmaceutical Resources Branch, Division of Clinical Investigation, National Cancer Institute, National Institutes of Health, Bethesda, MD. These materials were found to be at least 95% pure by ultraviolet absorbance and HPLC analysis. L-Heptanesulfonic acid, sodium salt, was a product of Eastman Kodak Co., Rochester, NY. Formic acid, 95-97%, was obtained from Aldrich Chemical Co., Milwaukee, WI. Deionized, glass distilled water which had been passaged through 0.2 μ membrane filters was used for the preparation of all solutions.

Blood samples (~2 ml) from the patient described were obtained from an indwelling catheter previously placed in the supe-

rior vena cava for other purposes. Plasma was obtained by centrifugation at 1000 xg for 10 min in a desk-top clinical centrifuge. One ml of the plasma was placed in a Centriflo CF-25 cone (25,000 MW cut-off, Amicon Corp., Lexington, MA), and the sample was centrifuged for 30 min at 900 xg in a Beckman Model J2-21 Centrifuge at 4°. The filtrate was injected onto a μBondapak C_{18} column (4mm x 30 cm; Waters Associates, Milford, MA) via a Rheodyne Injector (Model 7120, Rheodyne Incorporated, Berkeley, CA). The sample was eluted at 2 ml/min using a solution of 5mM 1-heptanesulfonic acid, adjusted to pH 2.4 with formic acid. A Laboratory Data Control (Riviera Beach, FL) Series 7800 Liquid Chromatograph was used. Detection of eluting materials was accomplished by ultraviolet absorbance at 254 nm (UV III Monitor) and at 280 nm (spectromonitor II). The flow of either of two Constametric Pumps was driven by a Chromatography Control Module II (CCM). The output of the two detectors was plotted and integrated simultaneously by the CCM, using the machine language programmed features of the instrument. The linear regression analysis and statistical calculations were performed using the BASIC programmable features of the CCM microprocessor as supplied by Mr. Mike Tarter, Laboratory Data Control, Rivera Beach,FL.

Urine samples were collected, placed on ice and an aliquot of the pretreatment (control) sample was "spiked" with

a working standard amount (10 μg/ml) of DON. Five ml aliquots of the urine samples were passaged through Sep-pak cartridges (Waters Associates) which had previously been washed with 5 ml of methanol, followed by at least 10 ml of distilled, deionized water. The urine samples were then filtered through 0.45 μ disposable membrane filters (Model SLHA 025 OS, Millipore Corp., Bedford, MA) and stored at -20° until analysis by HPLC. The informed consent of parents and patients was obtained for drug administration and for the sample collections from blood and urine.

RESULTS AND DISCUSSION

The reversed-phase, ion-pairing approach to HPLC is proving to be a very fruitful means of analysis of chemicals (5), particularly of drugs since many are either weak acids or weak bases. For analysis of DON, 1-heptanesulfonic acid was found to be suitable as an ion-pairing reagent (Figure 1). In experiments not shown, DON could be analyzed in plasma filtrates by elution of two reversed phase columns with a solution of 10 mM KH_2PO_4, pH 5.5; however, under these conditions separation of uric acid from DON was inadequate for drug measurement at levels less than 1 μg/ml. Retention of DON, but not uric acid, was increased by substituting 1-heptanesulfonic acid at pH 2.4 as the eluant as illustrated in Figure 1. Detection of DON at 254 nm and at 280 nm provides additional confirmation of the identity of the DON peak. Further confirmation can be made utilizing DON's temper-

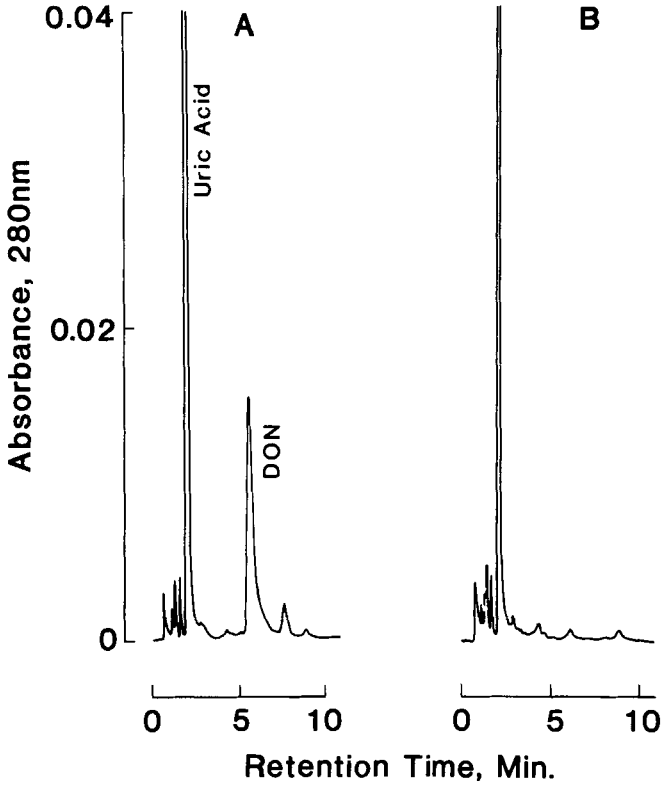

FIGURE 1. Representative separation of DON in human plasma. After removal of plasma proteins by membrane filtration, 50 µl of plasma containing 4 µg/ml of DON was injected onto the HPLC system described in Materials and Methods. A = sample prior to heat treatment. B = same sample after heating on a boiling water bath for 5 min.

ature instability, i.e., boiling the sample for 5 min eliminates the UV absorbing peak characteristic of DON (Figure 1).

A representative standard curve for DON in plasma is shown in Figure 2. Although the determination of DON in plasma samples containing 0.2 to 1.6 µg/ml is shown, additional determinations confirm linearity of detection to levels as

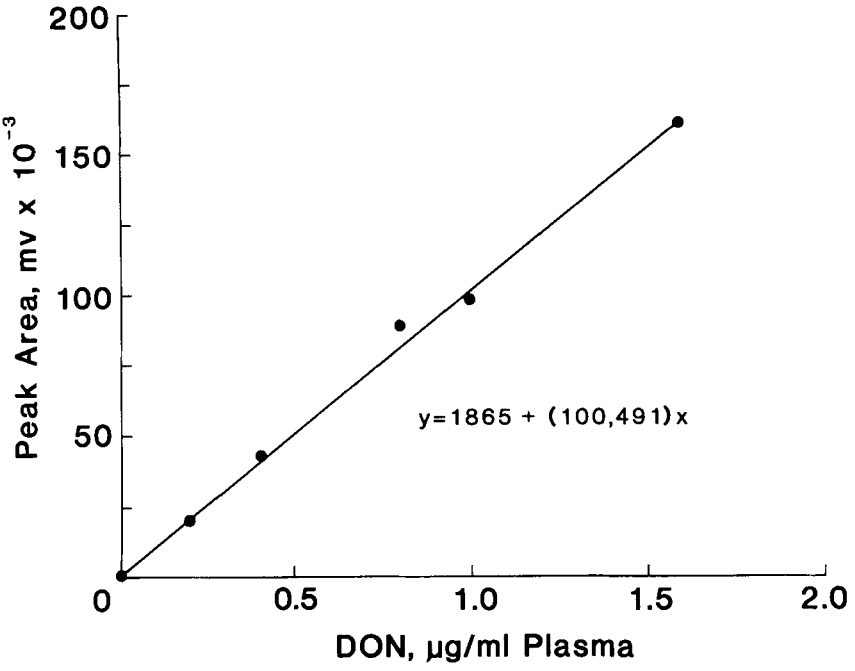

FIGURE 2. Standard curve for the HPLC analysis of DON in human plasma. The areas under the UV absorbing peaks at 280 nm are plotted at various known amounts of DON added to pooled human plasma. Qualitatively similar, but quantitatively different, curves were generated at 254 nm (data not shown). The equation shown is the least squares linear regression of the data.

high as 50 µg/ml. Table 1 presents data which indicates the quantitative recovery of DON during the membrane filtration step to remove plasma proteins, i.e., DON is not bound to plasma proteins. Also, the reproducibility of measurements of DON added to plasma is indicated in Table 1. DON was found to be stable in plasma prior to or after centrifugal membrane filtration when frozen at -20° for 1 week.

TABLE 1

Recovery of DON from Human Plasma Using a Membrane Filtration Method to Eliminate Proteins

Sample No.	DON, µg/ml	% Recovery
1	5.39	100
2	5.94	111
3	5.53	103
4	5.01	93
Mean value ± S.E.	5.47 ± 0.19	102 ± 3.7

DON was added to pooled human plasma to give a final concentration of 5.37 µg/ml before removal of plasma proteins and assay by HPLC as described in Materials and Methods.

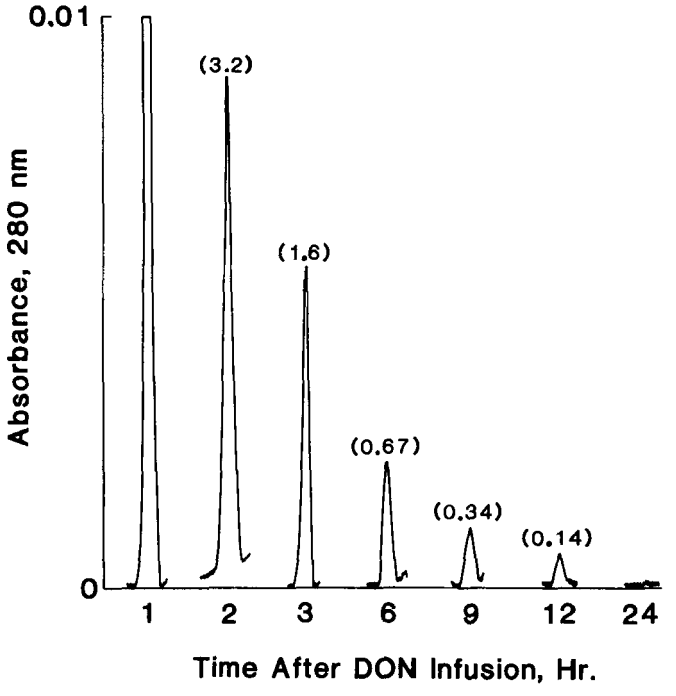

FIGURE 3. Disappearance of DON from the plasma of a child receiving 150 mg/m^2 as a 15 min infusion. The DON peak monitored at 280 nm is shown. The numbers in parenthesis are the integrated values for DON in plasma in µg/ml.

Application of the HPLC method for analysis of DON in plasma of a patient treated with the drug is given in Figure 3. The patient received 165 mg of DON (150 mg/m^2) as an i.v. infusion during a 15 min interval. Considerably less than 2 mg were excreted in the urine during the subsequent 24 hr collection interval in other pediatric patients treated with this dose (data not shown). The lower limit for detection of DON in urine is approximately 1 μg/ml.

In summary, previously reported methods for the analysis of DON in biological fluids have included microbiological (6,7), spectrophotometric (8) and HPLC after derivatization (9). Although the limits of sensitivity for some of these assays are adequate for the relatively-high doses being used in the current Phase I trials of the drug, the ease of sample preparation and the speed of the present method are important considerations for those interested in the clinical analysis of this rather labile chemical.

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