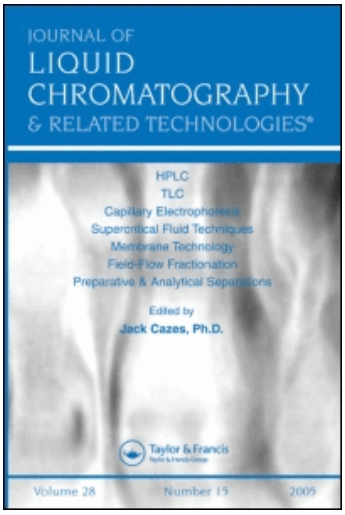


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

ISOCRATIC DETERMINATION OF DACARBAZINE AND RELATED IMPURITIES 2-AZAHYPOXANTHINE AND 5-AMINO-IMIDAZOLE-4-CARBOXAMIDE BY HPLC ON AN AVIDIN PROTEIN COLUMN

Ahsanul Haque[a]; James T. Stewart[a]
[a] Department of Medicinal Chemistry, College of Pharmacy, The University of Georgia, Athens, GA, U.S.A.

Online publication date: 22 March 1999

To cite this Article Haque, Ahsanul and Stewart, James T.(1999) 'ISOCRATIC DETERMINATION OF DACARBAZINE AND RELATED IMPURITIES 2-AZAHYPOXANTHINE AND 5-AMINO-IMIDAZOLE-4-CARBOXAMIDE BY HPLC ON AN AVIDIN PROTEIN COLUMN', Journal of Liquid Chromatography & Related Technologies, 22: 6, 933 — 943
To link to this Article: DOI: 10.1081/JLC-100101709
URL: http://dx.doi.org/10.1081/JLC-100101709

J. LIQ. CHROM. & REL. TECHNOL., 22(6), 933–943 (1999)

ISOCRATIC DETERMINATION OF DACARBAZINE AND RELATED IMPURITIES 2-AZAHYPOXANTHINE AND 5-AMINO-IMIDAZOLE-4-CARBOXAMIDE BY HPLC ON AN AVIDIN PROTEIN COLUMN

Ahsanul Haque, James T. Stewart*

Department of Medicinal Chemistry
College of Pharmacy
The University of Georgia
Athens, GA 30602-2352, USA

ABSTRACT

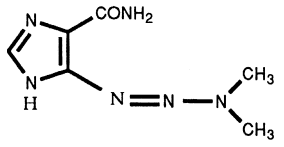
The separation of dacarbazine and its two related impurities, 5-amino-imidazole-4 carboxamide (AIC) and 2-azahypoxanthine (AHX) was investigated by capillary electrophoresis and high performance liquid chromatography. Baseline separation of dacarbazine and its related impurities was achieved by capillary electrophoresis (CE) on a fused silica capillary (70 cm x 50 μm, i.d) with an electrolyte buffer of methanol: phosphate buffer (0.025 M, pH 4) - 2:98 v/v at an applied voltage of 15 kV and detection at 220 nm. The CE method could not be used for the quantitation of impurities because at high concentration, the dacarbazine peak overlapped the nearest impurity peak. The initial HPLC method developed on an octadecylsilane (ODS) column successfully separated the impurities from the dacarbazine, but required a longer time of analysis (>50 min). Another HPLC method developed on an avidin protein column separated the drug and its impurities with a run time of less than 27 min and was chosen for method development and validation.

Dacarbazine and its two impurities were separated isocratically on avidin column using a mobile phase of 2-propanol: phosphate buffer (pH 7, 0.02 M) 4:96 v/v at a flow rate of 0.6 mL/min and detection at 230 nm. Calibration curves were prepared for each impurity from 100-1000 ng/mL. The detector response was linear (r^2 >0.997) for each individual impurity down to 100 ng/mL which represented 0.5% w/w of the dacarbazine concentration. Accuracy and precision of analysis for each impurity were less than 3 % at 100 ng/mL. The limits of quantitation and detection (s/n >3) for both impurities were 100 ng/mL and 50 ng/mL, respectively. Linearity was also demonstrated for dacarbazine in the 15-25 μg/mL range with a correlation coefficient greater than 0.998.

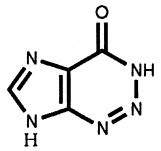
INTRODUCTION

Dacarbazine [5 (3,3 dimethyl -1-triazene)-imidazole 4-carboxamide] is an anti-neoplastic agent currently used in the treatment of melanoma, soft tissue sarcoma, neuroblastoma, and Hodgkin's disease in combination with other antineoplastic agents.[1] Dacarbazine alone is found to be effective in the treatment of malignant glucagonoma.[1] The drug is currently administered by i.v bolus or by i.v. infusion. Dacarbazine is sensitive to light and undergoes photolytic degradation to 2-azahypoxanthine (AHX).[2] In humans, dacarbazine undergoes N-demethylation by the liver microsomal enzymes to form 5-amino-imidazole-4-carboxamide (AIC). AIC is also considered to be the major metabolite in humans. Small amounts of dacarbazine can also form the diazonium salt of AIC, which undergoes intramolecular coupling to form AHX. It is assumed that some of the metabolites of dacarbazine also contribute to its antineoplastic activity.[1] Figure 1 shows the structures of dacarbazine, AIC and AHX.

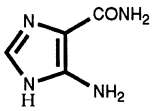
At present, there are only two analytical methods available for the simultaneous quantitation of dacarbazine and its related impurities (AIC and AHX). Both methods used HPLC and gradient elution techniques in their analysis.[3, 4] Fiore et al[3] reported a method for the determination of dacarbazine and AIC using an ODS column and gradient elution analysis with a total run time of 20.5 min. Tate et al[4] developed a method to determine the drug and AIC on a phenyl column using gradient analysis with a total run time of 13 min. The disadvantages of gradient elution analysis are the necessity of using two pumps and switching valves and substantially longer equilibration times which are required between analyses of multiple or replicate samples. The other reported methods for dacarbazine involved normal phase HPLC,[5] polarography,[6] electrochemistry,[7] and TLC.[8]



Dacarbazine



2-Azahypoxanthine (AHX)



5-amino -1 H imidazole-carboxamide (AIC)

Figure 1. Structures of dacarbazine and its impurities (AIC and AHX).

Stewart et al.[5] reported a method to determine dacarbazine along with other anticancer drugs by HPLC using underivatized silica. Barreira-Rodriguez et al.[7] determined dacarbazine and AIC by electrochemical detection. Kaleagasioglu[8] reported a TLC method to determine dacarbazine along with other anti-neoplastic agents. AIC and AHX are considered related impurities in the official USP 23 monograph for dacarbazine.[9] Dacarbazine is determined manually in USP 23 by recording absorbance in a suitable spectrophotometer at 323 nm and its impurities are quantitated by relative measurement of light intensity compared to dacarbazine on a TLC plate. The maximum USP allowable limits of each dacarbazine impurity is 1% w/w.

In this paper, an HPLC method is described to determine dacarbazine and its two related impurities under isocratic conditions on a commercially available avidin protein column. The packing contained an avidin protein covalently bound to a hydrophobically-derivatized silica surface.[10] This paper also discusses the separation of dacarbazine and its related impurities by capillary electrophoresis and by another HPLC method on an ODS column. Capillary electrophoresis is gaining increasing popularity in the area of impurity analysis because of its high efficiency and consumption of little or no organic solvent in the run buffer.

EXPERIMENTAL

Reagents and Chemicals

Dacarbazine, 5-amino-imidazole-4 carboxamide (AIC) and 2-azahypoxanthine (AHX) were donated by USP, Rockville, MD. All solvents were of HPLC grade and the reagents were of highest quality commercially available. Mobile phases were prepared using freshly distilled water and they were degassed before use. The Bioptic AV-1 avidin protein column (150 x 4.6 mm i.d) and its guard column (10x4.6 mm i.d) were obtained from Meta Chem Technologies (Torrance, CA). The Zorbax ODS column (250x 4.6 mm i.d) was purchased from Mac Mod Analytical (Chadds Ford, PA). Fused silica capillary (72 cmx 50μm i.d) was purchased from Polymicro Technologies Incorporated (Phoenix, AZ) for the electrophoretic investigations.

Instrumentation and Chromatographic Conditions

The HPLC system consists of a Beckman pump (Model 110A), an Alcott autosampler (Model 278) equipped with a 100 μL loop and a UV/Vis detector (Waters, Model 481). The chromatograms were recorded on a Shimadzu integrator (Model C-R3A, Chromatopac). All the separations were performed on the avidin column at a flow rate of 0.6 mL/min and at ambient temperature (23 ±1°C). The column was protected with a guard column containing the same stationary phase and a frit filter coupled on-line. The detection wavelength was set at 230 nm. Capillary electrophoresis (CE) was performed on a fused silica capillary (70 cmx 50 μm i.d) using an ABI CE instrument (Model 270 A) which was connected to a Hewlett Packard integrator (Model-3395). The sample was injected into the CE system in the hydrodynamic mode with a voltage of 15 kV at ambient temperature with detection at 220 nm.

Preparation of Standard Solutions for Calibration Curves

A stock solution of dacarbazine (500 μg/mL) was prepared in absolute methanol. Aliquots from the stock were spiked into 1 mL of mobile phase to prepare concentrations in the 15-25 μg/mL range for calibration curves. A stock solution of AIC (20 μg/mL) was dissolved in water and aliquots from the stock were added to the mobile phase to make 0.1 to 1 μg/mL concentrations for calibration curves. A stock solution of AHX (20 μg/mL) was prepared in methanol and aliquots from the stock were spiked into 1 mL of the mobile phase to make 0.1 to 1 μg/mL solutions. Fresh stock solutions were prepared daily and stored at 4°C. Spiked solutions were mixed pre-injection into the HPLC system.

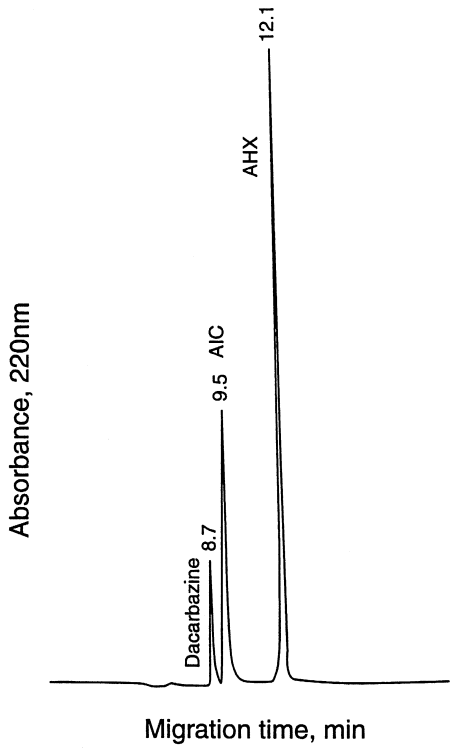


Figure 2. Electropherogram of dacarbazine and its two impurities, 5-amino-imidazole-4 carboxamide (AIC) and 2-azahypoxanthine (AHX) on a fused silica capillary (70 cm x 50 μm, i.d) with an electrolyte of methanol:phosphate buffer (pH 4, 0.025 M) - 2: 98 v/v at 15 kV and ambient temperature at 220 nm.

RESULTS AND DISCUSSION

Initially, the separation of dacarbazine and its related impurities was investigated by capillary electrophoresis (CE). The separation of drug and its impurities was achieved on a fused silica capillary with an electrolyte buffer of methanol: phosphate buffer (0.025M, pH 4) - 2:98 v/v at a voltage of 15 kV at ambient temperature. Figure 2 shows the electropherogram of the drug and its impurities. The migration times were 8.7, 9.5, and 12.1 min for dacarbazine, AIC, and AHX, respectively. The resolution between analytes was more than adequate to determine the drug and its impurities simultaneously, but the method could not be used for impurity determination at the 1% w/w level because the dacarbazine peak at higher concentration, overlapped the nearest eluting peak (AIC). Increased resolution between AIC and dacarbazine was observed at pH

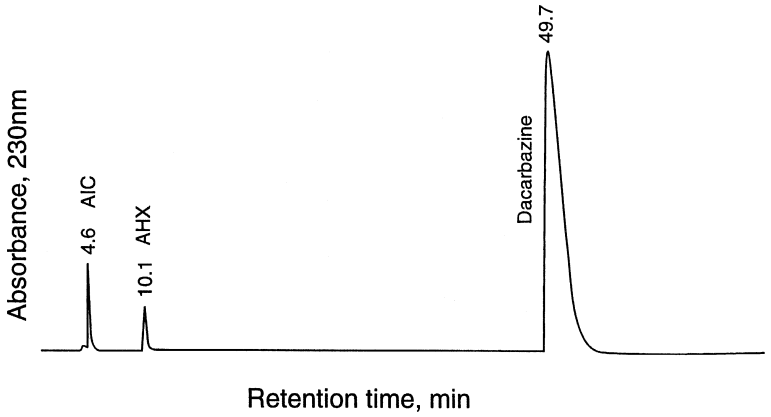


Figure 3. Chromatogram of dacarbazine and its two impurities, 5-amino-imidazole-4 carboxamide (AIC) and 2-aza hypoxanthine (AHX) on an ODS column by HPLC with a mobile phase of neat phosphate buffer (pH 2.5, 0.02M) at a flow rate of 0.6 mL/min, detection at 230 nm and at ambient temperature.

below 3, but the migration time of AHX was more than 60 min. Adjusting the buffer pH to 4 decreased the migration time of AHX to 12 min at the expense of resolution between dacarbazine and AIC. Attempts were not successful in CE to find a suitable electrolyte buffer to determine the drug and its impurities at the 1 % w/w impurity level either by manipulation of electrolyte composition such as pH, buffer types (phosphate, acetate, citrate), organic modifiers, or additives such as surface active agents or triethylamine.

In an initial HPLC study, the retention of dacarbazine and its impurities was investigated on a classic ODS column. Compared to dacarbazine, both AIC and AHX were weakly retained on the column. The retention times of AIC, AHX, and dacarbazine were 5, 10.5, and 102 min, respectively, with a mobile phase of neat phosphate buffer (pH 7, 0.02 M) at a flow rate of 0.6 mL/min. The retention times of AIC and AHX did not change significantly on the ODS column in the pH range of 2.5-7, however, the retention time of dacarbazine decreased as the pH was lowered. Addition of small amounts (1%) of either methanol, 2-propanol, or acetonitrile in the mobile phase reduced the retention time of dacarbazine, but it overlapped the peaks of AIC and AHX. Finally, a neat phosphate buffer (pH 2.5, 0.02 M) was selected to separate dacarbazine and its impurities on the ODS column with retention times of 4.6, 10.1, and 49.7 min for AIC, AHX, and dacarbazine, respectively, at a flow rate of 0.6 mL/min and ambient temperature (see Figure 3).

Due to the weak retention of AIC and strong retention of dacarbazine on the ODS column, the total run time could not be optimized less than 50 min. Thus, it was decided to investigate a commercially available avidin protein column for the method development because a protein column provides the scope for manipulation of the retention times of analytes either by changing the charge state of analytes or the protein stationary phase. In an avidin column, the packing material contains an avidin protein covalently bonded to an aminopropyl-derivatized silica surface. Avidin is a very basic protein, having an isoelectric point of 9.5-10.0. It is present in small amounts in egg-white and can be isolated in the pure state by electrophoresis without difficulty.[11] The protein is composed of four identical sub-units, each having a molecular weight of 16,400 and a completely known sequence. The protein has functional groups such as histidine, asparagine, and glutamine whose ionization state depends on the pH of the medium. The protein phase at various pHs unfolds and refolds, exposing new areas for interaction with analytes. With the avidin column, the retention of analytes depends on both ionic and hydrophobic interactions.[12] Other nonspecific interactions such as hydrogen bonding also contribute to the retention of the solute.[12]

The effect of mobile phase compositions on the retention of the drug and its impurities on the avidin column was investigated by varying pH (2.5-7.2), organic modifiers and phosphate buffer concentrations (0.1-1.5 M). Dacarbazine (protonated form, pKa 4.42) and its related impurities are basic in nature and vary in polarity. AIC is the most polar analyte followed by AHX and dacarbazine in order. The retention of the analytes was strongly influenced by the pH of the mobile phase. The retention of all three analytes increased as the pH increased because of hydrophobic interactions with the protein phase. At pH below 5, AIC eluted close to the solvent front. The pH of the mobile phase was adjusted to 7 to increase the retention of AIC on the column. With neat phosphate buffer (pH 7,0.02 M), retention times of AIC, AHX, and dacarbazine were 5.5, 50, and 70 min, respectively. To reduce the retention times of AHX and dacarbazine, various organic modifiers such as methanol, 2-propanol, and acetonitrile were investigated. Acetonitrile and 2-propanol gave similar retention times of the analytes on the avidin column, but selectivity of the analytes was different with methanol which provided the longest retention times. Four percent by volume of 2-propanol was added in the mobile phase (pH 7,0.02 M) to provide retention times of 4.5, 10.8, and 25.3 min for AIC, AHX, and dacarbazine, respectively. The retention of AIC did not decrease significantly with addition of the 4% 2-propanol in the mobile phase compared to neat phosphate buffer. The retention of AIC on the protein phase probably depends more on ionic or other nonspecific interactions than hydrophobic interaction. Changing the phosphate buffer concentration of the mobile phase from 0.02 to 1.5 M did not alter the analyte retention times significantly.

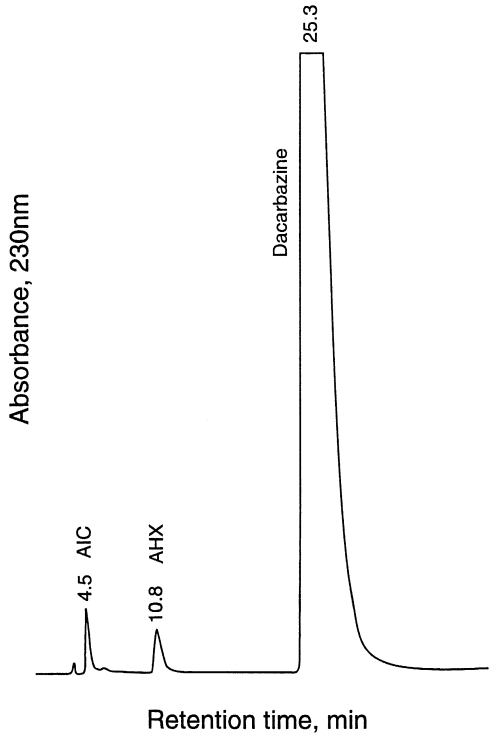


Figure 4. Chromatogram of dacarbazine and its two impurities, 5-amino-imidazole-4 carboxamide (AIC) and 2-aza hypoxanthine (AHX) on an avidin column by HPLC with a mobile phase of 2-propanol:phosphate buffer (pH 7, 0.02M) - 2: 98 v/v at a flow rate of 0.6 mL/min, detection at 230 nm and at ambient temperature.

Figure 4 shows the separation of dacarbazine and its related impurities (AHX and AIC) on the avidin protein column with a mobile phase of 2-propanol: phosphate buffer (pH 7, 0.02 M)- 4:96 v/v at a flow rate of 0.6 mL/min and detection at 230 nm.

Calibration curves for AIC on the avidin column were prepared in the 100-1000 ng/mL range (100 ng/mL represents 0.5 % w/w impurity in dacarbazine). Typical correlation coefficients were greater than 0.997 (n=5). Calibration curves were made for AHX in the 100-1000 ng/mL range (100 ng/mL represents 0.5 % w/w impurity in dacarbazine) and typical correlation coefficients were greater than 0.999 (n=5). Calibration curves were prepared in the 15-25 μg/mL range (75-125 % of nominal drug substance) for dacarbazine and correlation coefficients were greater than 0.994 (n=5).

Table 1

Typical Linear Regression Data for Dacarbazine, AIC, and AHX

Analyte	Conc. (μg/mL)	r^2	Slope	Intercept
Dacarbazine	15 - 25	0.994	295936	-865362
AIC	0.1 - 1.0	0.997	276	-3268
AHX	0.1 - 1.0	0.999	274	5789

Table 2

Percent Error and Precision (%RSD) of Spiked Samples of Dacarbazine, AIH, and AHX

Analyte	Conc. Added (μg/mL)	Conc. Found (μg/mL)[a]	Percent Recovery	% RSD
Dacarbazine	22.5	22.47±0.03	99.87	0.13
	15.0	15.48±0.24	96.80	1.55
AIC	0.10	0.10±0.001	100.0	1.0
	1.0	1.0±0.004	100.0	0.40
AHX	0.20	0.205±0.001	97.50	0.48
	0.80	0.804±0.023	99.50	2.86

[a] Mean ± standard deviation based on n = 3.

The %RSD of ten replicate injections of dacarbazine at 20 μg/mL was less than 3%. The linear regression parameters are listed in Table 1. The results for the spiked samples are listed in Table 2. The accuracy and precision of analysis of all three analytes were less than 3%. The limits of quantitation and detection (s/n >3) for both AIC and AHX were 100 ng/mL and 50 ng/mL, respectively. The interday and intraday accuracy and precision of the analysis were less than 5%. The retention times, resolution, and tailing factors of the peaks did not change significantly over a period of three months.

CONCLUSIONS

The isocratic HPLC method described herein on a avidin column can be useful for the assay of dacarbazine and its two related impurities in a single injection. The method showed good accuracy and precision (< 3%) for the quantitation of both impurities at the 1% w/w level of the principle drug. The response of dacarbazine was linear over the 15-25 μg/mL concentration range of the drug and the %RSD of replicate injections was less than 3%. The resolutions between the analyte peaks was excellent and did not change significantly over a period of six months. The method can be used either to study the degradation of dacarbazine or to determine the impurities at the 1 % w/w level or both. A CE method was also optimized for the separation of dacarbazine and its two impurities within 13 min, but the method is not useful for the quantitation of impurities because of overlapping peaks at high concentrations of dacarbazine. An ODS column was also investigated and was found to be very retentive for dacarbazine, but less retentive for its impurities thus requiring longer analysis times.

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Received July 20, 1998
Accepted August 7, 1998
Manuscript 4786

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