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Reversed-phase high-performance liquid chromatography of 4-(2-pyridyl)-1-piperazinethiocarboxylic acid 2-[1-(pyridyl)ethylidene]hydrazide dihydrochloride (NSC 348977), a synthetic thiosemicarbazone with antitumor activity

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Abstract

Reversed-phase HPLC conditions for the separation of 4-(2-pyridyl)-1-piperazinethiocarboxylic acid 2-[1-(pyridyl)-ethylidene]hydrazide dihydrochloride (NSC 348977, **I**), a synthetic thiosemicarbazone with antitumor activity, from mouse plasma have been investigated. Following denaturization and precipitation of the spiked plasma with acetonitrile, an aliquot of the supernatant was diluted with aqueous buffer and subjected to analysis on a Nova-Pak C_{18} column (150×3.9 mm I.D.) by isocratic elution with 50 m*M* aqueous potassium phosphate buffer (pH 6.8, containing 1 m*M* EDTA)–acetonitrile (60:40, v/v). The column effluent was monitored for UV absorption at 310 nm. Problems identified in the sample preparation and separation of **I** include sensitivity to oxygen, light, non-neutral pH and the presence of metal ions. These factors were seen to adversely influence sample recovery, and attempts were made to find conditions which minimize their effects.

Keywords: NSC 348977; Thiosemicarbazone

1. Introduction

There are many literature reports of the chemotherapeutic effects of thiosemicarbazones [1–4]. Among the earliest was the use of thiosemicarbazones in the late 1940s for the treatment of tuberculosis. Structure–antitubercular activity has subsequently been investigated for thiosemicarbazone derivatives [5]. Encouraged by the demonstrated activity, investigators tested these substances for possible activity against other pathogenic microorganisms, notably pox viruses. The earliest observation of the antileukemic activity of thiosemicarbazones was by Brockman et al. [6]. Since that time, certain thiosemicarbazones have been shown to possess a wide range of actions, including fungistatic, anti-inflammatory, antiparasitic (*Trichomonas*

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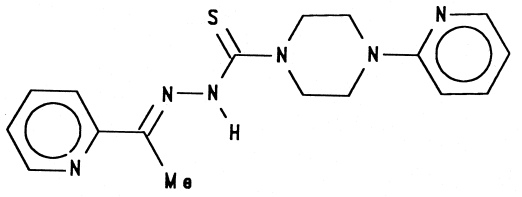
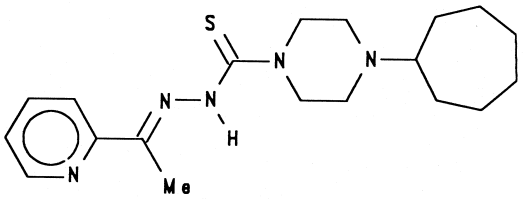
vaginalis), antiviral (vaccinia, herpes, cytomegalo) and antitumor activities [7] (and references therein).

Thiosemicarbazones have also been shown to be efficient tridentate ligands for many metal cations, forming octahedral complexes [8], and hence have found extensive use as spectrophotometric reagents in the analysis of metals [9]. Complexes of Cu(II), Ni(II) or Mn(II) with certain 2-acetylpyridine thiosemicarbazones showed reduced antimalarial activity in mice infected with *Plasmodium berghi*, but possessed enhanced antileukemic properties when compared to the free ligand [10]. Iron complexes of certain thiosemicarbazones have been shown to be significantly more active than the free ligands as inhibitors of ribonucleoside diphosphate reductase to which no iron had been added [11]. Interestingly, metal (other than iron) complexes of certain 2-acetylpyridine thiosemicarbazones showed reduced antimalarial activity but showed increased antileukemic activity [10].



(I)



(II)

Fig. 1. Structures of NSC 348977 (**I**) and the recovery enhancer NSC 376265 (**II**).

In the course of screening compounds for specific activity, the National Cancer Institute became interested in thiosemicarbazones in general, and in 4-(2-pyridyl)-1-piperazinethiocarboxylic acid 2-[1-(pyridyl)ethylidene]hydrazide dihydrochloride (NSC 348977, **I**) [9,11–20] (Fig. 1) in particular. This compound is a synthetically-derived thiosemicarbazone which has demonstrated significant activity against prostate cancer. Despite the wealth of available information and continued general interest in these compounds, there exists a conspicuous absence of reports dealing with the chromatographic behavior of these compounds, thereby inhibiting development of analytical methodology for determining these compounds in biological fluids. Reported herein are the results of our preliminary investigations into the development of such methodology.

2. Experimental

2.1. Materials and chemicals

4-(2-Pyridyl)-1-piperazinethiocarboxylic acid 2-[1-(pyridyl)ethylidene]hydrazide dihydrochloride (NSC 348977, **I**) and 4-(1-cycloheptyl)-1-piperazinethiocarboxylic acid 2-[1- (pyridyl)ethylidene]hydrazide dihydrochloride (NSC 376265, **II**) were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Mouse plasma was obtained from Harlan (Indianapolis, IN, USA). Ethylenediamine tetraacetic acid (EDTA) disodium salt solution (100 m*M*) and all other reagents were obtained from Aldrich (Milwaukee, WI, USA) and used as received. Distilled water was deionized and stripped of dissolved organics by passage through activated carbon and mixed bed resins (Hydro Service and Supplies, Rockville, MD, USA). All glassware was thoroughly cleaned, dried and then treated with SurfaSil (Pierce, Rockford, IL, USA) to deactivate surfaces.

2.2. Preparation of solutions

2.2.1. Mobile phase for reversed-phase high-performance liquid chromatography (HPLC)

The aqueous portion of the mobile phase (a 50 m*M* potassium phosphate buffer, pH 6.8, containing 1 m*M* ethylenediamine tetraacetic acid disodium

salt) was prepared by mixing aqueous solutions of potassium dihydrogenphosphate (25 ml of a 1 *M* solution), dipotassium hydrogenphosphate (25 ml of a 1 *M* solution) and 100 m*M* ethylenediamine tetraacetic acid disodium salt (10 ml), and then diluting the resulting mixture to a volume of 1 l by the addition of water. This solution and acetonitrile (60:40, v/v) were premixed and continuously sparged with helium before and during use.

*2.2.2. Stock solution of **I***

A stock solution of **I** in water was prepared by dissolving 1.0 mg **I** in 1.0 ml water, and was then maintained at −20°C when not in use. A working solution of **I** (20 μg/ml in water) was prepared daily as follows. HPLC-grade water was sonicated for 15 min then kept in an ice bath for at least 10 min before use. The stock solution was allowed to thaw at ambient temperature and then thoroughly mixed. A 30-μl aliquot was removed and added to 1.47 ml of water. The resulting mixture was vortexed for 15 s then kept in an ice bath until used.

*2.2.3. Stock solution of **II** (used as a recovery enhancer)*

A stock solution of **II** in acetonitrile was prepared by dissolving 2.5 mg of **II** in 10.0 ml of acetonitrile, and was then maintained at −20°C when not in use. A working solution of **II** (50 μg/ml) was prepared daily as follows. Aqueous 50 m*M* phosphate buffer (pH 6.6) was sonicated for 15 min then kept in an ice bath for at least 15 min before use. The stock solution was allowed to thaw at ambient temperature and then thoroughly mixed. A 100-μl aliquot was removed and added to 400 μl of the aqueous buffer solution, pH 6.6. The resulting mixture was vortexed for 15 s and kept in an ice bath until used. The 50 m*M* buffer (pH 6.6) was prepared by mixing aqueous solutions of potassium dihydrogenphosphate (34 ml of a 1 *M* solution) and dipotassium hydrogenphosphate (16 ml of a 1 *M* solution) and then diluting the resulting mixture to a volume of 1 l by the addition of water.

2.2.4. Stock solution of 4-chloro-1-naphthol (used as an internal standard)

A stock solution of 4-chloro-1-naphthol, the internal standard (I.S.), was prepared by dissolving the compound in mobile phase to give a concentration of 50 μg/ml. When not in use, this solution was protected from light and stored at ambient temperature.

2.3. Sample preparation

At the start of each day 15-ml portions of acetonitrile, 100 m*M* EDTA solution, and pH 6.8 buffer (as used in the preparation of the mobile phase) were placed in separate 130 mm×22 mm I.D. glass bottles fitted with sintered glass bubbling tubes (Ace Glass, Vineland, NJ, USA) and continuously sparged with helium. Mouse plasma (50 μl) was placed in 0.5-ml conical polypropylene centrifuge tubes (Eppendorf, Westbury, NY, USA) and a 1–10 μl portion of the working solution of **I** was added. The resulting mixture was vortexed for 5 s, whereupon addition of 10 μl of the working solution of **II** was followed by another 5 s of vortex mixing. A 10-μl aliquot of the I.S. solution of 4-chloro-1-naphthol was added and the mixture vortexed for 5 s. Next, EDTA (10 μl of a 100 m*M* solution) was added and the mixture subjected to vortex mixing for 15 s. Following the addition of acetonitrile (200 μl), the mixture was vortexed for 15 s and centrifuged for 5 min at 16 000 *g*. The supernatant (150 μl) was transferred to a 300-μl silanized borosilicate glass autosampler injection insert. After addition of 150 μl of the previously prepared pH 6.8 buffer solution, the contents were mixed by twice inverting the capped glass insert. Finally, a 250-μl aliquot of this solution was immediately subjected to analysis.

2.4. Liquid chromatography

HPLC was performed at ambient temperature using an HP Series 1050 pump and autosampler fitted with a 500-μl sample loop (Hewlett-Packard, Palo Alto, CA, USA). Samples were subjected to analysis by isocratic elution with a premixed and vigorously and continuously helium-sparged mobile phase (vide supra) at a flow-rate of 1 ml/min through a stainless steel 4 μm Nova-Pak C_{18} column (150× 3.9 mm I.D.) (Waters Associates, Milford, MA, USA) which was preceded by a stainless steel Brownlee NewGuard RP-18 guard column (15 mm× 3.2 mm I.D.) (Applied Biosystems, Foster City, CA, USA) and a 0.5-μm inlet filter. The column effluent was monitored for UV absorbance at 310 nm, and a

computing integrator was used to obtain retention time and peak area. Typical retention times for **I**, 4-chloro-1-naphthol (I.S.), and **II** were 5.7, 9.9, and 11.6 min, respectively. UV absorption spectra were recorded using a Model 8452A UV–Vis diode array spectrophotometer (Hewlett-Packard).

2.5. Quantitation

Once conditions for sample preparation and analysis had been identified such that replicate determinations were consistent, then attempts were made to construct standard curves in order to assess the possibility of quantitating **I** in plasma. Thus, standard curves were constructed by plotting the chromatographic peak area ratio of **I**/I.S. against the analyte concentration in the plasma standards. Linear least squares regression was performed unweighted and without inclusion of the origin to determine the slope, *y*-intercept and correlation coefficient of the best fit line.

3. Results and discussion

3.1. Sample recovery

Initial investigation of **I**, which was supplied as the dihydrochloride, revealed that the compound is quite soluble in water (14.7 mg/ml), DMSO and polar organic solvents, but relatively insoluble in non-polar organic solvents, e.g., hexane (0.021 mg/ml). In solution, **I** is chemically stable when maintained near neutral pH. The pH of maximum stability was determined to be 7.1 (half-life 70 h at 25°C in the dark). When exposed to extremes of pH, **I** suffered rapid and complete decomposition. The degradation of **I** was also accelerated when exposed to light.

Initial attempts to develop a liquid chromatographic assay found a tendency of **I** to complex with iron, either in solution or on equipment surfaces. In fact, it proved possible to form the iron complex, which is readily chromatographed, by adding a solution of $FeSO_4$ to the sample mixture. Unfortunately, the complex could not be induced to form in plasma and so this ''derivatization'' was not deemed a practical approach for the determination of **I**. However, adding 1 m*M* EDTA to the aqueous portion of the mobile phase gave highly reproducible chromatography and linear calibration curves for **I**.

Extraction of **I** from mouse plasma into an organic solvent followed by evaporation of the solvent under a stream of nitrogen resulted in low and highly variable recoveries. Attempts at solid-phase extraction using a 50 mg C_{18} SepPak (Waters Associates) also resulted in highly variable recoveries. Furthermore, larger sample tubes and longer vortexing times gave lower recoveries. It became evident that **I** was not only sensitive to pH and lighting conditions, but to the presence of oxygen as well. Thus, sparging solutions with helium to remove oxygen, using small (0.5-ml) centrifuge tubes, and keeping vortexing times to a minimum were critical to obtaining consistent recovery. To further enhance the recovery of small amounts of **I**, it was necessary to add another thiosemicarbazone, **II** (Fig. 1), as a recovery enhancer. It is thought that **II** may consume traces of oxygen and/or complex with traces of metals or active sites in the plasma, the sample preparation apparatus, or the chromatographic system. Although the exact function is not presently known, the addition of **II** results in a higher recovery of **I**. Typically, recovery of 0.06 μg of **I** from 50 μl of mouse plasma was 49%, but it was 76% when 0.5 μg of **II** was present. Recovery of **I** at low concentrations was further increased by the addition of EDTA to the plasma before the **I** was extracted into acetonitrile.

Thus, using small (0.5-ml) sample tubes, helium-sparged solutions, minimizing exposure to light, and adding **II** and EDTA resulted in high, reproducible recoveries of **I** from plasma. Extraction should be performed as rapidly as possible on one sample at a time, and, to minimize sample decomposition the acetonitrile extract should not be evaporated but diluted with buffer and immediately analyzed.

*3.2. Stability of **I** in mouse plasma*

Samples of mouse plasma containing **I** at 0.8, 2.0 and 4.0 μg/ml were prepared and 50-μl aliquots were placed in 0.5-ml polypropylene tubes and immediately frozen and stored at −78°C. Tubes were periodically removed, allowed to thaw for 3 min at room temperature, then processed and analyzed as

rapidly as possible as described above. Samples were thawed after 0, 1, 2, 4, 7, 11, 32 and 84 days. There was no evidence that **I** degraded while in plasma at −78°C.

In a separate series of experiments, mouse plasma containing **I** at a concentration of 3.3 μg/ml was allowed to stand at room temperature while aliquots were periodically removed, processed and analyzed. After 6.6 h, 92.1% of **I** remained. From these experiments, a t_{90} of 7.2 h is predicted for **I** in mouse plasma at ambient temperature.

3.3. Standard curves

UV detection was initially performed at 248 nm, which is the absorption maximum for **I** in mobile phase (Fig. 2). However, when samples in mouse plasma were analyzed, much less interference from extractables was encountered by using a wavelength of 310 nm instead. The cleaner chromatograms (Fig. 3) more than compensated for the reduced sensitivity at 310 nm.

A number of standard curves were constructed using concentrations of 0.4, 0.8, 1.2, 2.0, 2.8 and 4.0 μg/ml of **I** in 50 μl of mouse plasma using 4-chloro-1-naphthol as an I.S. The small sample volume was necessary in anticipation of having to perform replicate determinations of the drug in plasma obtained from a single mouse in actual pharmacokinetic studies. Standard curves were constructed using three determinations at each concentration. The analyte to I.S. chromatographic peak area ratio was directly proportional to the added concentration of **I** from approximately 0.4 to 4.0 μg/ml. Mean values (±S.D.) of the linear regression parameters for six

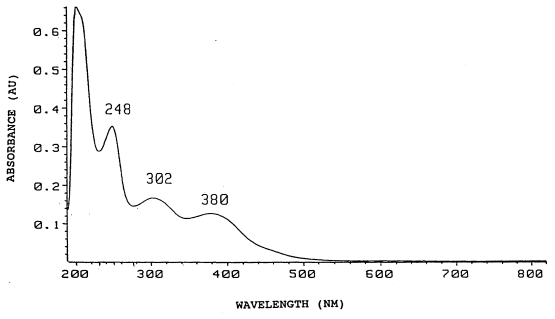


Fig. 2. UV–Vis spectrum of 10 μ*M* **I** in mobile phase.

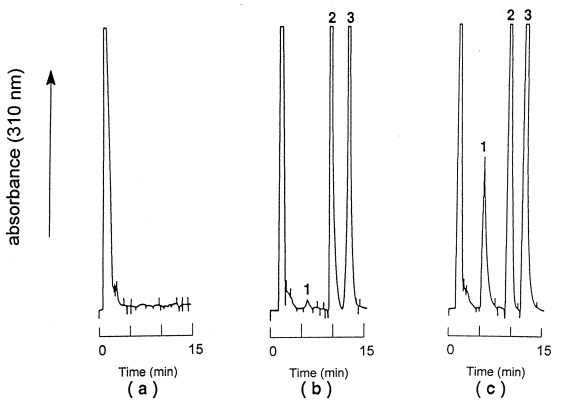


Fig. 3. Chromatograms of extracts of (a) blank plasma, (b) 50 μl plasma containing 0.02 μg **I** (0.40 μg/ml) and (c) 50 μl plasma containing 0.2 μg **I** (4.0 μg/ml). Peaks: 1=**I**; 2=4-chloro-1-naphthol (internal standard); 3=**II** (recovery enhancer).

standard curves of NSC 348977 in plasma, independently prepared and assayed over a two week period, were: slope, 0.06989±0.00584 ml/μg; *y*-intercept, −0.01139±0.00109; correlation coefficient, 0.9974±0.0014.

The limit of detection was 0.005 μg in 50 μl of plasma (0.1 μg/ml) based on a signal-to-noise ratio of 3:1. At concentrations below 0.02 μg in 50 μl of mouse plasma (0.4 μg/ml) peak area integration became increasingly variable. At this level, frequent replacement of the column inlet filter was necessary for reliable low-level detection. Whenever this filter was replaced, it was found necessary to pump mobile phase through it for several hours to remove any trace amounts of iron which may be present. In addition, new guard columns required conditioning by passage of mobile phase for several hours followed by several 10-μl injections of a 20 μg/ml solution of **I** prior to actual use.

Relative recovery and reproducibility data for the determination of **I** in mouse plasma are given in Table 1. The overall recovery (mean±S.D.) of **I** was 75.2±13.6% (n=108) and the overall recovery of 4-chloro-1-naphthol was 96.6±3.1% (n=108).

4. Conclusions

Problems identified in sample preparation and chromatographic separation of **I** include sensitivity to

Table 1
Relative recovery and reproducibility of the analytical method for the quantitation of **I** in mouse plasma

Amount added (μg/ml)	No. of replicates	Mean amount found (μg/ml)	Relative recovery	C.V. (%)
0.40	6	0.27	68.1	9.65
0.80	6	0.55	69.7	2.44
1.20	6	0.91	75.5	3.73
2.00	6	1.63	81.7	2.16
2.80	6	2.38	85.2	1.10
4.00	6	3.50	87.4	2.02

oxygen, light, non-neutral pH and the presence of metal ions. These factors were seen to adversely affect sample recovery. However, conditions were found which allowed reproducible recovery of **I** in the concentration range of 0.4 to 4.0 μg/ml from a 50-μl aliquot of mouse plasma. Future directions include using and refining (inter alia, extending the upper and lower concentration limits of the present standard curve) this preliminary methodology to determine time–plasma concentration levels of **I** in the mouse once a non-toxic dose has been established.

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References

[1] D. Hambre, J. Bernstein, R. Donovick, Proc. Soc. Exper. Biol. Med. 73 (1950) 275.
[2] K.A. Brownlee, D. Hambre, J. Bacteriol. 61 (1951) 127.
[3] D. Hambre, K.A. Brownlee, R. Donovick, J. Immunol. 67 (1951) 305.
[4] R.L. Thompson, S.A. Minton, J.E. Officer, G.H. Hitchings, J. Immunol. 70 (1953) 229.
[5] E. Saripinar, Y. Güzel, S. Patat, I. Yildirim, Y. Akçamur, A.S. Dimoglo, Arzneim.-Forsch. 46 (1996) 824.
[6] R.W. Brockman, J.R. Thomson, M.J. Bell, H.E. Skipper, Cancer Res. 16 (1956) 167.
[7] H.R. Wilson, G.R. Revankar, R.L. Tolman, J. Med. Chem. 17 (1974) 760.
[8] M. Mathew, G.J. Palenik, J. Am. Chem. Soc. 91 (1969) 6310.
[9] R.B. Singh, H. Ishii, Crit. Rev. Anal. Chem. 22 (1991) 381.
[10] J.P. Scovill, D.L. Klayman, C.F. Franchino, J. Med. Chem. 25 (1982) 1261.
[11] L.A. Saryan, E. Ankel, C. Krishnamurti, D.H. Petering, H. Elford, J. Med. Chem. 22 (1979) 1218.
[12] D.L. Klayman, J.P. Scovill, J.F. Bartosevich, C.J. Mason, J. Med. Chem. 22 (1979) 1367.
[13] A.S. Dobek, D.L. Klayman, E.T. Dickson, J.P. Scovill, E.C. Tramont, Antimicrob. Agents Chemother. 18 (1980) 27.
[14] D.L. Klayman, J.P. Scovill, J.F. Bartosevich, C.J. Mason and R.S. Griffin, US Pat., 4 317 776 (1982).
[15] C. Lambrow, G.E. Childs, J.D. Notsch, J.P. Scovill, D.L. Klayman, D.E. Davidson, Antimicrob. Agents Chemother. 22 (1982) 981.
[16] F.M. Collins, D.L. Klayman, N.E. Morrison, J. Gen. Microbiol. 128 (1982) 1349.
[17] R.A. Casero, D.L. Klayman, G.E. Childs, J.P. Scovill, R.E. Desjardins, Antimicrob. Agents Chemother. 18 (1980) 317–322.
[18] C. Shipman, S.H. Smith, J.C. Drach, D.L. Klayman, Antiviral Res. 6 (1986) 197.
[19] D.L. Klayman, A.J. Lin, J.W. McCall, S.-Y. Wang, S. Townson, M. Grogl, K.E. Kinnamon, J. Med. Chem. 34 (1981) 1422.
[20] D.L. Klayman, J.P. Scovill, C.J. Mason, J.F. Bartosevich, J. Bruce, A.J. Lin, Arzneim.-Forsch. 33 (1983) 909.