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High-performance liquid chromatographic method for guanylhydrazone compounds

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Abstract

A high-performance liquid chromatographic method has been developed for a series of aromatic guanylhydrazones that have demonstrated therapeutic potential as anti-inflammatory agents. The compounds were separated using octadecyl or diisopropyloctyl reversed-phase columns, with an acetonitrile gradient in water containing heptane sulfonate, tetramethylammonium chloride, and phosphoric acid. The method was used to reliably quantify levels of analyte as low as 785 ng/ml, and the detector response was linear to at least 50 μg/ml, using a 100 μl injection volume. The assay system was used to determine the basic pharmacokinetics of a lead compound, CNI-1493, from serum concentrations following a single intravenous injection in rats.

Keywords: Guanylhydrazones; CNI-1493

1. Introduction

Septic shock syndrome is a frequent complication of critical illness, and is associated with an increased mortality rate. This syndrome is due to an over-production of endogenous cytokines, nitric oxide, and other mediators by host monocytes/macrophages in response to pathogenic stimuli, such as bacterial endotoxin [1,2]. Experimental compounds which competitively inhibit nitric oxide synthase (such as N^G-methyl-L-arginine and N^G-methyl-L-arginine methyl ester) actually exacerbate the mortality of the shock state [3]. Recent evidence has shown that this increase in mortality is due to the nonspecific inhibitory activity of such arginine analogues for both cytokine-inducible macrophage nitric oxide synthesis and constitutive synthesis in blood vessels [4]. This latter activity, termed endothelium-derived relaxing factor (EDRF), is necessary to relieve local vasoconstriction of blood vessels, and inhibition during shock increases mortality [4].

Recently, it has been demonstrated that certain aromatic oligo-guanylhydrazones, related to compounds originally synthesized as anti-trypanosomal agents [5], could selectively inhibit cytokine-inducible macrophage nitric oxide syn-

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thesis without inhibiting EDRF activity [6]. In mouse models, one particular compound, CNI-1493 (I), was found to protect against endotoxic shock and also prevented carrageenan-induced inflammation [6]. In order to further study the pharmacological properties of these anti-inflammatory compounds, it was necessary to design an accurate and sensitive assay system. We report here the development of an ion-pair, reversed-phase high-performance liquid chromatographic (HPLC) method and its application in the analysis of test compounds in biological samples. While HPLC methods exist for the quantification of methylglyoxal-bis-guanylhydrazone [7–11] and guanabenz([(2,6 - dichlorobenzylidene)amino]-guanidine) [12,13], the present method represents the first described assay for aromatic compounds with multiple-guanylhydrazone groups.

2. Experimental

2.1. Chemicals

Heptane sulfonate (HS), tetramethylammonium chloride (TMAC), and phosphoric acid were obtained from Aldrich (Milwaukee, WI, USA), and pentamidine isethionate from May and Baker (now Rhone-Poulenc; Dagenham, UK). HPLC-grade acetonitrile was acquired from Fisher (Fairlawn, NJ, USA) and all water was filtered and deionised by a Picopure system (Hydro Service and Supplies; Research Triangle Park, NC, USA). All guanylhydrazones were synthesized as described [5,14] and the purity confirmed by elemental analysis, ^{1}H-NMR, and melting point.

2.2. Chromatographic conditions

A Hewlett-Packard Model 1090 liquid chromatograph (Wilmington, DE, USA) equipped with an autosampler, photodiode-array detector, and Chemstation operating software was used for all analyses. The columns used were either a Supelcosil LC-18 250 × 4.6 mm octadecylsilane column with 5 μm particle size (Supelco, Bellefonte, PA, USA) or a Zorbax RX-C8 250 × 4.6 mm column with 5 μm particle size (Mac Mod Analyticals, Chadds Ford, PA, USA) kept at room temperature. Buffer A was 10 m*M* HS–10 m*M* TMAC–4.2 m*M* H_3PO_4/H_2O, and buffer B 10 m*M* HS–10 m*M* TMAC–4.2 m*M* H_3PO_4–75% CH_3CN–25% H_2O. Using a flow-rate of 1.5 ml/min, runs were initiated at 10% B and a linear gradient to 90% B was performed over 30 min. The column was then returned to 10% B over 7 min, followed by 3 min re-equilibration. The compounds were detected by absorbance at 265 nm, with 540 nm used as a reference wavelength.

2.3. Sample preparation

The test compounds and the internal standard, pentamidine (see Fig. 1), were dissolved in distilled water to make 1 mg/ml stock solutions. To determine the relative retention times and peak shapes, a single test compound and the internal standard were diluted to 10 μg/ml in distilled water, and 100 μl injected onto the HPLC. Standard addition curves were constructed in distilled water, human urine, and mouse serum by the addition of various amounts of test compound and 5 μg/ml pentamidine.

2.4. Pharmacokinetic studies

Male Sprague–Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, USA) were anaesthetised with ketamine and the right carotid artery cannulated with polyethylene tubing (PE-50). The animals were given 10 mg/kg of I in a single intra-arterial push injection of 380 μl. At 0, 5, 15, 30, 60, 90, 120, 180, 240, 300, 360 min 400 μl of blood was removed, stored at 4°C for 4 h, and then centrifuged at 15 000 *g* for 10 min, and the serum layer collected. Sodium azide was added to 0.01% v/v to prevent microbial growth and pentamidine was added to 5 μg/ml. Twenty-five μl of the resulting sample was injected onto the HPLC.

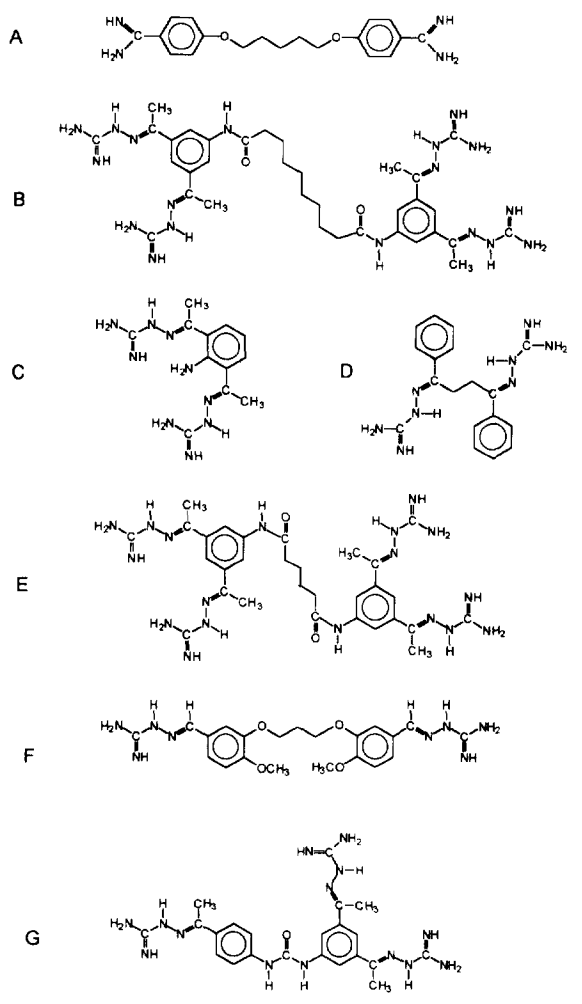

Fig. 1. The structures of the compounds used in this study. (A) Pentamidine [1,5-bis(4′-amidinophenoxy)pentane], the internal standard. (B) CNI-1493 [N,N′-bis(3,5-diacetylphenyl)decanediamide-tetrakis(amidinohydrazone)] (I). (C) CNI-2993 [2,6-diacetylanaline-bis(amidinohydrazone)]. (D) CNI-2093 [1,4-diphenyl-1,4-butanedione-bis(amidinohydrazone)]. (E) CNI-2293 [N,N′-bis(3,5-diacetylphenyl)-hexanediamide-tetrakis(amidinohydrazone)]. (F) CNI-1893 [3,3′-(trimethylenedioxy)-di-*p*-anisaldehyde-bis(amidinohydrazone)]. (G) CNI-993 [N-(4-acetylphenyl)-N′-(3,5-diacetylphenyl)urea-tris(amidinohydrazone)].

3. Results and discussion

In designing a separation system for the aromatic guanylhydrazones, ion-pair buffers were chosen which contained 10 m*M* heptane sulfonate, 10 m*M* tetramethylammonium chloride, and 4.2 m*M* phosphoric acid, as these buffers had been used successfully to separate aromatic diamidines [15], which bear some structural similarity to the present compounds. Use of these buffers with a reversed-phase C_{18} column was found to be ideal for the elution of I. Elimination of either of the ion-pair reagents from the buffer led to a complete retention of I by the HPLC column (data not shown). Initially, separation of I and the internal standard, pentamidine, was performed with a Supelcosil LC-18 column, which provided excellent separation and peak shape (Fig. 2A). However, the separation on more recent Supelcosil LC-18 columns was found to be much poorer (Fig. 2B), and a Zorbax RX-C8 column was substituted (Fig. 2C). Using the Zorbax RX column, all of the guanylhydrazone compounds were found to be resolved from each other and the internal standard, with excellent peak shape (Fig. 3).

Further studies with I demonstrated that the limit of detection was 785 ng/ml for a 100-μl injection. The assay was found to be linear from the limit of detection up to at least 50 μg/ml, and gave the following regression for a plot of I peak area vs. ng/ml injected: $y = 2675x - 452$ ($r^2 = 1.00$). The method was also found to be accurate, with an intra-day variation of 1.5% on samples of 10 μg/ml I ($n = 4$), and an inter-day variation of 9.5% on samples of 6.25 μg/ml ($n = 3$).

The HPLC method was applied towards estimating the pharmacokinetic parameters of I in adult rats receiving a 10 mg/kg dose as a single intra-arterial, push injection. In these experiments, the solid-phase extraction step was omitted due to the small volume of each sample, and the relatively large amount of I which was recovered. Typical serum decay curves were obtained (Fig. 4, solid line), and the method of residuals was used to calculate the pharmacokinetic parameters (Fig. 4, dashed lines). The distribution rate constant (α) was found to be 0.31 ± 0.09 min^{-1}, the elimination rate constant (β) $2.30 \times 10^{-3} \pm 0.00 \times 10^{-3}$ min^{-1}, the initial distribution concentration (A) 63.01 ± 43.78 μg/ml, the initial elimination concentration (B) 1.57 ± 0.14 μg/ml, the distribution half-life

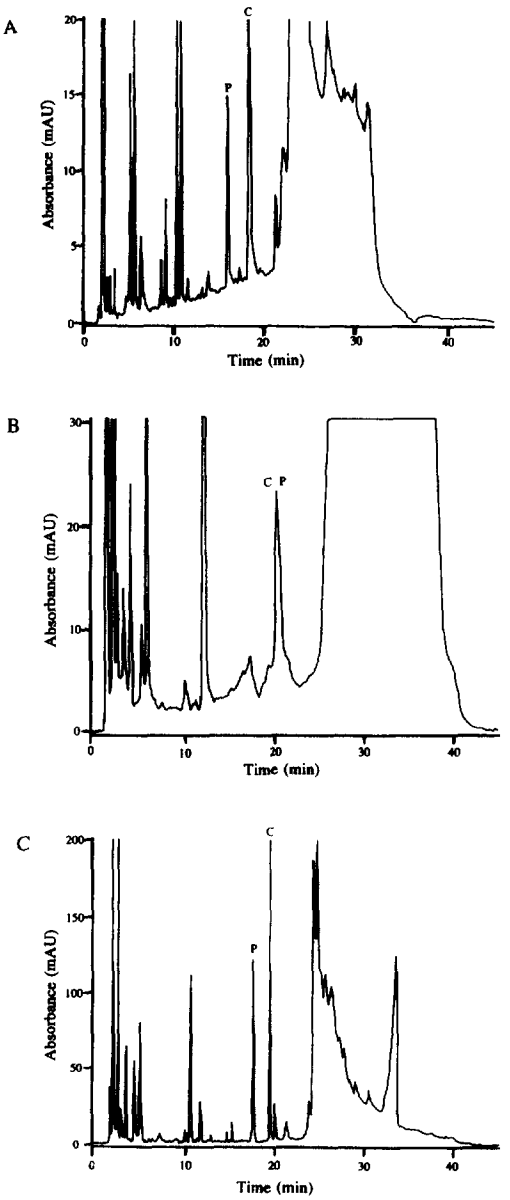

Fig. 2. The chromatographic separation of I from mouse serum. A total of 6.5 μg/ml I and 5.0 μg/ml pentamidine were added to samples of mouse serum, before injection of 100 μl and separation using the conditions described in Section 2. The columns used for the separations were: (A) a Supelcosil LC-18 250 × 4.6 mm column purchased in 1987; (B) a Supelcosil LC-18 250 × 4.6 mm column purchased in 1995; and (C) a Zorbax RX-C8 250 × 4.6 mm column purchased in 1995. The peaks labelled P and C refer to pentamidine and I, respectively.

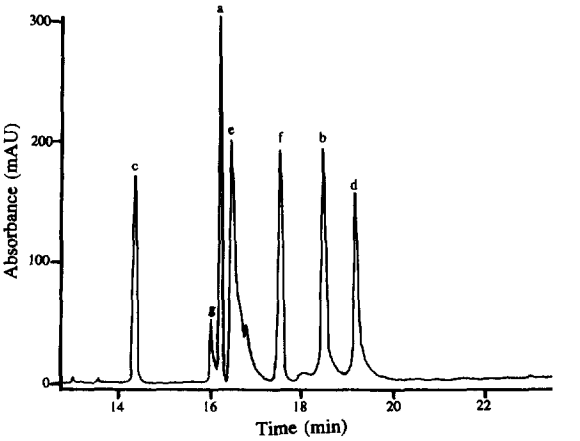

Fig. 3. The chromatographic separation of several aromatic guanylhydrazones injected simultaneously. Each compound was added to distilled water to a final concentration of 10 μg/ml, and then 100 μl injected onto a Zorbax RX-C8 column using the conditions described in Section 2. The labels on each peak correspond to the structures given in Fig. 1.

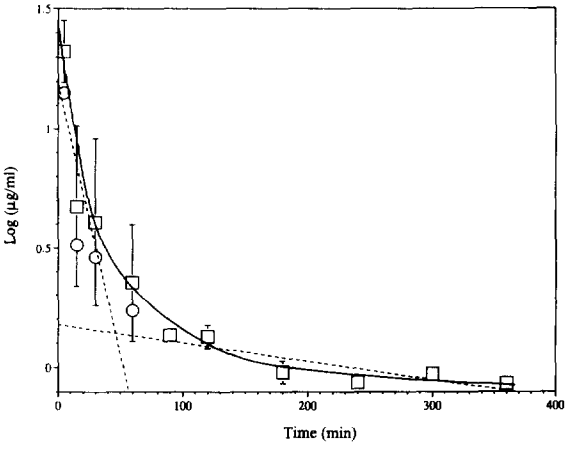

Fig. 4. The serum concentrations of I in rats following a single intravenous injection. 10 mg/kg injections were made into three animals according to the procedure described in Section 2, and then sequential blood samples were taken. A total of 25 μl of each serum sample was injected and the amount of I quantified. The graph represents the time course of I disappearance from the blood (solid line), and the extrapolated distribution and elimination phases (dashed lines), as determined by the method of residuals [17]. Each square point represents the average ± standard deviation for three rats, and each circular point the corresponding calculated distribution phase residual point. The pharmacokinetic values calculated from this graph are presented in the text.

($t_{1/2}\alpha$) 2.41 ± 0.69 min, the elimination half-life ($t_{1/2}\beta$) 5.02 ± 0.00 h, the volume of distribution (V_d) 2.45 ± 0.21 l, and the total clearance (C_L) 5.62 ± 0.47 ml/min ($n = 3$ for all). These values show that the compound persists in the serum for some time after a single i.a. injection. Experiments performed via intraperitoneal or oral dosing routes indicate that the drug is not rapidly absorbed, and may have a low bioavailability (data not shown). The choice of a 10 mg/kg dose is applicable, as the compound was found to have an LD_{50} of 70 mg/kg when given intraperitoneally and one that exceeds 1 g/kg when given orally.

Aromatic bis-guanylhydrazones similar to the ones described were first synthesized in 1984 [5], and have since been utilised in a number of disease models [6,16]. To date this report is the first which describes an HPLC assay system for this class of compound. With the recent discovery that these compounds exhibit considerable activity in animal models of septic shock [6], there will be an increased need for systems to detect and quantify aromatic guanylhydrazones in biological samples. The sensitivity and accuracy of the method outlined here indicate that it should be ideal for such purposes, and preliminary studies using the HPLC system to study the pharmacokinetics of specific lead compounds appear to support this conclusion.

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References

[1] K.J. Tracey and S.F. Lowry, Adv. Surg., 23 (1990) 21.
[2] D.S. Brett and S.H. Snyder, Ann. Rev. Biochem., 63 (1994) 175.
[3] E. Nava, R.M.J. Palmer and S. Moncada, Lancet, 1 (1991) 1555.
[4] J.P. Cobb, C. Natanson, W.D. Hoffman, R.F. Lodato, S. Banks, G. Koer, M.A. Solomon, R.J. Elin, J.M. Husseini and R.L. Danner, J. Exp. Med., 176 (1992) 1175.
[5] P. Ulrich and A. Cerami, J. Med. Chem., 27 (1984) 35.
[6] M. Bianchi, P. Ulrich, O. Bloom, M. Meistrell, G.A. Zimmerman, H. Schmidtmayerova, M. Bukrinsky, T. Donnelly, R. Bucala, B. Sherry, K.R. Manogue, A.J. Tortolani, A. Cerami and K.J. Tracey, Mol. Med., 1 (1995) 254.
[7] H.M. Wallace and G.A. Cameron, J. Chromatogr., 380 (1986) 122.
[8] D.L. Gildersleeve, M.C. Tobes and R.B. Natale, Clin. Chem., 31 (1979) 1979.
[9] M.G. Rosenblum and T.L. Loo, J. Chromatogr., 183 (1980) 363.
[10] J. Roboz, T. Konrad and R.D. Hart, J. Anal. Toxicol., 4 (1980) 127.
[11] K.C. Marsh, A.J. Repta and L.A. Sternson, J. Chromatogr., 187 (1980) 101.
[12] L. Vio, M.G. Mamolo and G. Furlan, Farmaco, 43 (1988) 27.
[13] J.A. Knowles, G.R. White, C.J. Kick, T.B. Spangler and H.W. Ruelius, J. Pharm. Sci., 71 (1982) 710.
[14] P. Ulrich, R.W. Grady and A. Cerami, Drug Dev. Res., 2 (1982) 219.
[15] B.J. Berger, V.V. Reddy, S.T. Le, R.J. Lombardy, J.E. Hall and R.R. Tidwell, J. Pharmacol. Exper. Ther., 256 (1991) 883.
[16] F.W. Jennings, P. Ulrich and A. Cerami, Trop. Med. Parasitol., 38 (1987) 181.
[17] M. Gibaldi and D. Perrier, Pharmacokinetics, Marcel Dekker, New York, 1982, pp. 433–444.