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Determination of the antileukemic drug mitoguazone and seven other closely related bis(amidinohydrazones) in human blood serum by high-performance liquid chromatography

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Abstract

A reversed-phase (C_{18}) HPLC method with diode-array detection was developed for the separation and determination of methylglyoxal bis(amidinohydrazone) (mitoguazone) and seven closely related aliphatic analogs thereof, namely the bis(amidinohydrazones) of glyoxal, dimethylglyoxal, ethylmethylglyoxal, methylpropylglyoxal, butylmethylglyoxal, diethylglyoxal and dipropylglyoxal. The mobile phase consisted of a non-linear binary gradient of methanol and 0.03 *M* aqueous sodium acetate buffer (pH 4.3). Good separation of the eight congeners was achieved. On increasing the methanol content of the eluent, the bis(amidinohydrazones) eluted in the order of increasing number of carbon atoms in the side-chains. The method was also applied to the quantitative analysis of the compounds in aqueous solution and, combined with ultrafiltration, for the separation of the eight congeners in spiked human blood serum. A separate simplified method for the quantitative determination of each of the compounds in spiked human blood serum samples was also developed. The methods developed made for the first time possible the simultaneous HPLC analysis of more than one bis(amidinohydrazones). The results obtained indicate that the bis(amidinohydrazones) studied obviously have a distinct tendency to form ion associates with acetate ions and probably also other carboxylate ions in aqueous solution. This aspect may be of biochemical significance, especially concerning the intracellular binding of the compounds. Each one of the compounds studied invariably gave rise to one peak only, this result supporting the theory that the conventional synthesis of each of the compounds gives rise to one geometrical isomer only. This result is completely in agreement with the results of previous proton and carbon NMR spectroscopic as well as X-ray diffraction studies.

Keywords: Mitoguazone; Bis(guanylhydrazones); Bis(amidinohydrazones)

1. Introduction

Glyoxal bis(amidinohydrazone) (GBG) and its methylglyoxal analog (MGBG, mitoguazone) are potent antileukemic agents whose biochemical and pharmacological properties have been subject to intensive research (for nomenclature and abbreviations as well as for structures, see Table 1) [1–10]. In the case of MGBG, a large number of clinical studies have also been carried out [5,11–23]. In these studies, MGBG has been used either alone or in combination with α-difluoromethylornithine, an ir-

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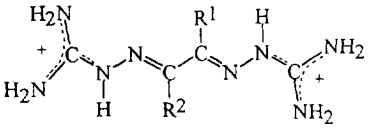
Table 1
Structures of the bis(amidinohydrazones) studied



Compound	R_1	R_2
GBG	H	H
MGBG	CH_3	H
DMGBG	CH_3	CH_3
EMGBG	CH_2CH_3	CH_3
DEGBG	CH_2CH_3	CH_2CH_3
MPGBG	$CH_2CH_2CH_3$	CH_3
BMGBG	$CH_2CH_2CH_2CH_3$	CH_3
DPGBG	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$

Bis(amidinohydrazones) are commonly called 'bis-(guanylhydrazones)'. Therefore, their established abbreviations contain the letters GBG, preceded by an indication of the substituent (e.g. DE for diethyl). The term 'bis(amidino-hydrazone)' is, however, more appropriate. The Chemical Abstracts' systematic name for GBG is 2,2′-(1,2-ethanediylidene)bis(hydrazinecarboximidamide).
The formula represents the structures of the di-cation forms of the compounds.

reversible inhibitor of ornithine decarboxylase (ODC), but also other combination chemotherapy regimens have been employed.

GBG and MGBG are also potent specific inhibitors of S-adenosylmethionine decarboxylase (AdoMetDC), one of the two key enzymes of polyamine biosynthesis (the other one being ODC), and they interfere with polyamine metabolism also by inhibiting diamine oxidase (DAO) and by entering cells via the putative polyamine carrier [1–7,9,10,24–28]. Because of these properties, other bis(amidinohydrazones) and related compounds have received much attention as well. Several novel analogs have been synthesized, some of which are far more potent inhibitors of AdoMetDC than GBG and MGBG [2–7,28–32]. Among aliphatic congeners, the bis(amidinohydrazones) of ethylmethylglyoxal and diethylglyoxal (EMGBG and DEGBG, respectively) are especially interesting in this respect [2,6,7].

Because the compounds are highly valuable pharmacological tools, reliable methods for their analysis in biological samples are needed. Although some HPLC methods [33–37] and one gas chromatographic-mass spectrometric method [38] have been reported for the analysis of MGBG, methods for other congeners have been few. One HPLC method has been published for the analysis of phenylglyoxal bis(amidinohydrazone) [39]. A biochemical method, based on the inhibition of AdoMetDC and DAO by the compounds, has been employed for many congeners [40], but this method cannot be used if the sample contains more than one congener, or if it contains inhibitors of AdoMetDC or DAO other than bis(amidinohydrazones). The only method reported for the simultaneous analysis of several bis(amidinohydrazones) has been micellar electrokinetic capillary chromatography [41,42].

In this paper, we report an HPLC method for the separation of a total of eight different aliphatic bis(amidinohydrazones), including GBG and MGBG as well as various mono-and dialkylglyoxal analogs thereof (for the compounds studied, see Table 1). Methods for the quantitation of the compounds are also reported. The methods, combined with ultrafiltration, have also been applied for the analysis of the compounds in spiked human blood serum.

2. Experimental

2.1. Chemicals

All of the bis(amidinohydrazones) studied were in the free base form and were synthesized according to previously published procedures [2,6,7,9,29].

Analytical grade acetic acid and anhydrous sodium acetate were obtained from E. Merck (Darmstadt, Germany). Methanol was either from E. Merck (LiChrosolv gradient grade) or from Rathburn (Walkerburn, UK) (HPLC grade). Water was purified by using the Gelman Water-I system (Ann Arbor, MI, USA) or the Millipore Alpha-Q system (Bedford, MA, USA).

2.2. HPLC apparatus and conditions

Two chromatographic systems were used in this study. Most measurements, including method development, were performed with a Hewlett-Packard (HP, Avondale, PA, USA) HPLC system consisting of a HP Model 1090 instrument equipped with a

Model 1040 diode-array detector and a Rheodyne Model 7125 manual loop injector (Rheodyne, Cotati, CA, USA) with a 5-μl sample loop. An HP 85B computer was used for data acquisition and processing. In the HP apparatus, the solvents were mixed at low pressure.

Some measurements were also carried out using a Shimadzu HPLC system consisting of two LC-10AD liquid chromatograph solvent delivery systems and an SPD-M6A photodiode-array UV–Vis detector (Shimadzu, Kyoto, Japan). A manual Rheodyne Model 7125 loop injector with a 5-μl sample loop was used for injecting the samples. Data processing was performed using Shimadzu program SPD-M6A version 2.24. In the Shimadzu apparatus, the mixing chamber was a high-pressure type one, three different mixing volumes being available (0.5, 1.7 and 2.6 ml). In the analyses performed, the smallest chamber volume gave optimal results and was invariably used.

A column (100×2.1 mm I.D.) packed with Nucleosil 100-10 C_{18} material (10 μm mean particle size) was employed throughout the study. In the case of serum ultrafiltrates, either a LiChrosorb RP-18 precolumn (25×4.6 mm I.D., particle size 5 μm) or a LiChroCART 4-4 precolumn containing LiChrospher 100 RP-18 packing material (particle size 5 μm) was used, the former with the HP apparatus and the latter with the Shimadzu apparatus. Both precolumns were obtained from E. Merck.

Separations were carried out at ambient temperature by using a binary gradient system with methanol and aqueous sodium acetate buffers (pH 4.3) as the constituents of the mobile phase. In most cases, the concentration of the acetate buffer was 0.03 *M*. The gradients used are described in detail in Table 2. A constant flow-rate of 1.0 ml/min was used.

Values of t_0 were determined by injecting 5 μl of 0.03 *M* aqueous $NaNO_3$. For the HP system the t_0 values obtained were 0.6 min (with precolumn) and 0.5 min (without precolumn).

The pilot signal used for recording two-dimensional chromatograms with the diode-array detectors was 282 nm, the band width being 4 nm. UV–Vis spectra were acquired at the apex as well as the ascending and descending part of each peak.

2.3. UV spectrometry

UV spectra were recorded with a Perkin-Elmer 554 spectrophotometer (Norwalk, CT, USA). The absorption maxima of the bis(amidinohydrazones) were studied using solutions of the compounds (about 0.1 mg/ml) in the 0.03 *M* acetate buffer or in the same buffer containing 5% methanol.

2.4. Blood samples, separation of serum and preparation of spiked samples

Human blood was taken by venous puncture from an apparently healthy 24-year-old male volunteer. The blood was collected in vacuum glass tubes of 10 ml size (Venoject, Leuven, Belgium), and the serum

Table 2
Gradients used for the separations

Method	Linear step, methanol content from 0% to 8% (min)	Isocratic step, methanol content 8% (min)	Linear step, methanol content from 8% to 20% (min)
A	0–5.0	5.0–7.9	7.9–8.0
B	0–5.0	5.0–9.4	9.4–9.5
C	0–5.0	5.0–9.0	9.0–9.1
D	0–5.0	5.0–10.5	10.5–10.6
E	0–6.5	6.5–	–
F	0–6.5[a]	6.5–	–

Gradients A–D are for the separation of the eight bis(amidinohydrazones) in aqueous solutions as well as in serum ultrafiltrates, while E and F are for quantitation of the compounds in serum ultrafiltrates (E for GBG, MGBG, DMGBG, EMGBG and DEGBG; F for MPGBG, BMGBG and DPGBG). A=HP system without precolumn; B=HP system with precolumn; C=Shimadzu system without precolumn; D=Shimadzu system with precolumn. F and G are applicable for both systems with precolumns.

[a] Increase of methanol content from 0% to 20%.

was separated according to conventional methods. Serum was stored frozen at ca. −25°C.

Before chromatographic analyses, unspiked serum as well as bis(amidinohydrazone)-spiked serum samples were ultrafiltered using Centrisart I SM 13229E tubes (Sartorius, Göttingen, Germany) that were centrifuged at room temperature first for 5 min at 500 *g* and then for further 55 min at 1000 *g*. With this method, ca. 500 μl of a colourless ultrafiltrate were obtained from a 1-ml sample. When recovery percentages were determined, 2-ml samples were used instead, yielding slightly less than 1 ml of the ultrafiltrate.

When the linearity of the quantitative methods developed was tested for blood samples, bis(amidinohydrazone)-spiked serum samples were prepared by adding 100 μl of a fresh aqueous solution of the appropriate bis(amidinohydrazone) to 900 μl of thawed serum in the outer tube of the Centrisart two-tube system and by mixing the components with the aid of a 1-ml Finnpipette (Labsystems, Helsinki, Finland). When recoveries of the bis(amidinohydrazones) from serum were investigated, a more sophisticated procedure, in which sources of error such as foaming were minimized, was found to be necessary in order to improve the repeatability. Thus, the spiked samples were prepared by adding 200 μl of the aqueous solution to a 2-ml volumetric flask (grade A) and by filling the flask with serum. In order to assure mixing of the components, the flasks were turned upside-down fifty times, this procedure being performed for a total of three times during the 30-min period between spiking and ultrafiltration.

3. Results and discussion

3.1. Detection of compounds: UV absorption

Divalent salts of GBG and MGBG are known to absorb light strongly in the UV region, their spectra containing an absorption maximum at about 280 nm [9]. This phenomenon was verified in the present study, all of the bis(amidinohydrazones) studied being found to absorb strongly in the UV region near 280 nm when dissolved in the 0.03 *M* sodium acetate buffer. The absorption intensities of the various congeners, however, varied considerably and the absorption maxima occurred at slightly different wavelenghts (GBG 282 nm; MGBG 285 nm; DMGBG, EMGBG and DEGBG 279 nm; MPGBG, 280 nm; BMGBG, 280 nm; DPGBG, 281 nm). The addition of 5% of methanol to the buffer solutions did not significantly alter the wavelengths of the absorption maxima.

In a study on the UV spectroscopy of GBG [43], we have found that the shape and the absorption maxima of the UV spectrum of GBG free base in water change remarkably as a function of concentration. This phenomenon is obviously due to changes in species distribution and indicates that if aqueous solvents are used as eluents in HPLC analyses of bis(amidinohydrazones), they must be buffered. When spectra of the analytes were recorded with a diode-array detector during chromatographic runs, the absorption maxima remained unchanged even when the methanol content of the mobile phase was increased to 30%. Thus, UV absorption offers a feasible method for the detection of the compounds under study.

3.2. Method development for the separation of the bis(amidinohydrazones)

Some procedures for the HPLC analysis of MGBG have been reported. In most of them, specific ion-pair reagents are employed [33,35–37,39]. One relatively simple method has been reported by Rosenblum and Loo [34] who used an ODS (μBondapak C_{18}, 300×4 mm I.D.) column as the stationary phase. Their method was isocratic, the mobile phase being a 0.03 *M* sodium acetate buffer (pH 4.3) containing 5% methanol. No specific ion-pair reagent was used. At first, we tested, whether a modification of that method [34] could be used for the separation of the eight closely related bis(amidinohydrazones). Previous studies by our group [44] have indicated that in aqueous solution at pH 4.3, the species distributions of the bis(amidinohydrazones) of various mono- and dialkylglyoxals are essentially similar to each other, the compounds existing practically exclusively in the di-cation form. At somewhat higher pH values (e.g. 6.5), the species distributions of various congeners, however, differ clearly from each other. According-

ly, throughout the present study, we used mobile phases whose pH was 4.3. Thus, any complications caused by the presence of basic forms could be avoided. Initially, we used only a 0.03 *M* sodium acetate buffer (pH 4.3) as the mobile phase, methanol being omitted. We also used a somewhat different column. Separate experiments were performed for each of the bis(amidinohydrazones). When this isocratic method was employed, the congeners eluted in the order of the number of carbon atoms in the side chains, DEGBG eluting before MPGBG (Fig. 1). The retention times of GBG, MGBG, DMGBG and EMGBG were relatively short (1.6–15.3 min) and the shapes of their peaks relatively good although some tailing occurred, while DEGBG and MPGBG gave quite broad peaks and their retention times also were too long to be acceptable. MBGBG gave no detectable peak although elution was continued for 1 h, and therefore no attempt was done to analyze the even more highly alkylated congener DPGBG with the aid of the isocratic method.

Thus, an isocratic method with the 0.03 *M* acetate buffer alone as the mobile phase clearly was not applicable for the separation of the congeners, although it could obviously be used for the analysis of the least-substituted congeners. The effect of buffer concentration was then studied using MGBG as a model compound. The retention time of MGBG was slightly decreased (from 3.5 to 3.0 min) when the concentration of the buffer was lowered from 0.03 to 0.01 *M*. The decrease of the retention time being so small, it was obvious that a change of the concentration of the buffer probably would not alone have resulted in any significant improvement. Even a relatively small increase of the concentration of the buffer (from 0.03 to 0.1 *M*) in turn clearly increased the retention time of MGBG that was more than doubled to 8.1 min. With the longer retention time, the peak became broader without any marked decrease of tailing.

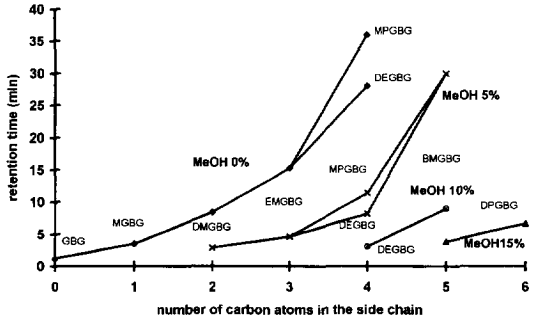


Fig. 1. Retention times of the bis(amidinohydrazones) studied, shown as a function of the total number of carbon atoms in the side-chains. Each of the compounds (70–100 μg/ml) was analysed separately by isocratic elution with a 0.03 *M* aqueous sodium acetate buffer (pH 4.3) containing 0–15% of methanol (MeOH). Separation conditions in Section 2.

The results obtained clearly indicated that specific ion-pair reagents are not necessary when bis(amidinohydrazones) are analysed by reversed-phase HPLC at a pH as low as 4.3, at which the bis(amidinohydrazones) are known to exist practically exclusively in the di-cation form [44]. The observation that the retention times of the bis(amidinohydrazones) depend on the concentration of the acetate buffer and the concentration of the analyte, indicates that bis(amidinohydrazones) have a tendency to form ion associates with acetate ions in the aqueous solutions used. This makes possible the separation of bis(amidinohydrazones) without an added ion-pair reagent.

Addition of methanol to the mobile phase was found to remarkably decrease the retention times of the bis(amidinohydrazones) and also to significantly sharpen the peaks. In Fig. 1, the effect of the number of side-chain carbon atoms on the retention time is shown for eluents with different methanol contents.

Based on the results of the above experiments, a binary gradient method (method A; see Table 2) was constructed for the separation of the eight bis(amidinohydrazones) studied. In this method, a non-linear gradient of the 0.03 *M* sodium acetate buffer (pH 4.3) and methanol is employed, elution starting with the buffer alone, the concentration of methanol being increased in a linear fashion from 0 to 8% between 0 and 5.0 min. Then, an isocratic step (8% methanol) results, after which the methanol content is again linearly increased to finally become 20%. Depending on the conditions and apparatus used, the method had to be slightly modified by changing the duration of the isocratic step (see Table 2 for details). The main principle of the method, however, worked well with both the HP apparatus and the Shimadzu apparatus. In the Shimadzu apparatus, the volume of the mixing chamber can be varied. We found that optimal results were obtained

by using the smallest possible volume. By using the larger volumes, DEGBG and MPGBG could not be separated.

Typical chromatograms of standard mixtures containing approximately equal amounts of each one of the bis(amidinohydrazones) either in aqueous solution or in a serum ultrafiltrate are shown in Fig. 2. The retention time of each analyte was found to depend to some extent on the concentration of the analyte, the retention time being increased on decreasing the concentration (Fig. 3). The retention times were also found to depend considerably on the temperature, becoming shorter on increasing the temperature (data not shown). The separation of DEGBG and MPGBG (both of which are equally highly alkylated congeners) is the most difficult point of the method and, therefore, the timing of the end of the isocratic step must in each case be be adjusted according to the concentration range of the analytes and the temperature. Because the method employed

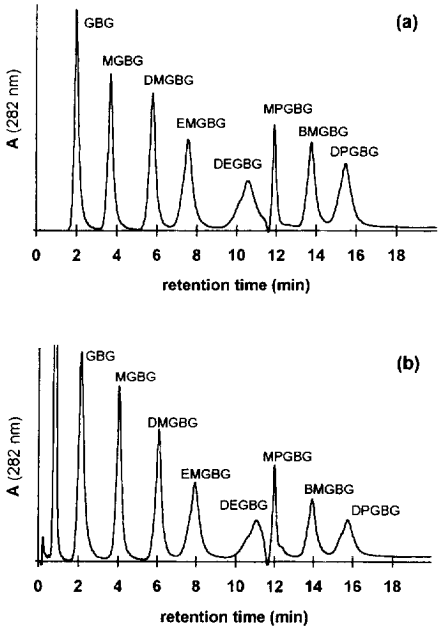


Fig. 2. Chromatograms of gradient elutions (gradient D) of a standard mixture containing 12.5 μg/ml of each of the eight bis(amidinohydrazones) studied. The one shown in (a) was obtained from a mixture of the bis(amidinohydrazones) in aqueous solution and the one shown in (b) from a serum ultrafiltrate. In the chromatograms of the ultrafiltrates of unspiked serum, the only detectable peaks were the ones observed before GBG. Separation conditions in Section 2.

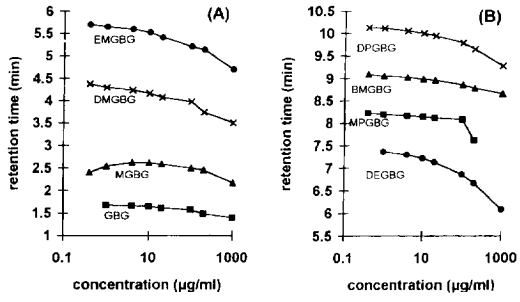


Fig. 3. Retention times of the bis(amidinohydrazones) as a function of concentration. The samples were analysed using gradient A. Separation conditions in Section 2.

self-packed narrow-bore columns, column-to-column variation may also be very large.

For determination of bis(amidinohydrazones) in human blood serum, two simplified linear gradient modifications (methods E and F in Table 2) of the above non-linear gradients were also developed. Thus, GBG, MGBG, DMGBG, EMGBG and DEGBG were analysed in serum using a gradient (method E), in which the percentage of methanol increased from 0 to 8% in 6.5 min from the start. In order to sharpen the peaks and to increase sensitivity, MPGBG, BMGBG and DPGBG were analysed using a steeper gradient (method F). In this gradient, the percentage of methanol increased from 0 to 20% in 6.5 min.

3.3. Quantitative analyses

The above non-linear gradient method A was applied also to the quantitation of the bis(amidinohydrazones) in aqueous solution. The limit of detection (defined as $S/N=3$) was approximately 0.04 μg/ml for GBG and 0.4 μg/ml for all other congeners except DEGBG, whose limit of detection was somewhat higher (about 1 μg/ml) because of the considerable broadening of its peak. Broadening can be effectively avoided by increasing the concentration of methanol but then DEGBG and MPGBG cannot be separated. When compared with the published MECC method [41], the detection limits of the present HPLC method are far lower (0.04 μg/ml vs. 1 μg/ml in the case of GBG). By MECC, better resolution is obtained, but the resolution obtained by the present HPLC method is yet

good enough to separate the bis(amidinohydrazones) from each other in aqueous solution as well as in serum ultrafiltrates.

The linearity of method A was tested separately for each compound using aqueous solutions of free bases whose concentrations ranged between 0.4 and 1000 μg/ml (for DEGBG and MPGBG, the concentration ranges were 1.0-1000 μg/ml and 0.4-100 μg/ml, respectively). The regression analysis (absorbance versus concentration) of each bis(amidinohydrazone) provided a correlation coefficient of 0.999 or higher (n=6–9; duplicate analysis run in each case). In the case of methods E and F that were used for the quantitation of the bis(amidinohydrazones) in serum ultrafiltrates, the concentration range studied for linearity was 2.0–50 μg/ml ($r \geq 0.996$, n=4; duplicate analysis run in each case), except for DEGBG for which it was 10–50 μg/ml (r=1.000, n=3; duplicate analysis run in each case). The low solubility of DPGBG allowed only a qualitative analysis of the compound in spiked serum samples.

One monoalkyl congener (MGBG) and one dialkyl congener (EMGBG) were selected for studies on the recovery of bis(amidinohydrazones) from spiked human blood serum (Table 3). In these studies, two different concentration levels were employed. With the lower analyte level (10 μg/ml), no marked loss of the analyte was observed. When the concentration of the analyte increased to 50 μg/ml, the recovery, however, decreased somewhat, the reasons for this phenomenon being obscure. The repeatability of the method used was good. R.S.D. values for samples in aqueous solutions varied between 2.7% and 5.6%. When the compounds were analysed from serum ultrafiltrates, the R.S.D. values were between 3.5% and 3.9% (see Table 3 for details).

3.4. Implications concerning the isomerism and the biochemical properties of bis(amidinohydrazones)

In principle, the synthesis of each one of the symmetrical bis(amidinohydrazones) now studied (i.e., GBG, DMGBG, DEGBG and DPGBG) can yield three different geometrical isomers, and the synthesis of each unsymmetrical congener (MGBG, EMGBG, MPGBG and BMGBG) four different isomers. The isomerism of bis(amidinohydrazones) is of considerable biochemical and pharmacological interest since different isomers would probably have different biochemical properties. If different isomers were formed in the syntheses of the compounds, the drastic biochemical and pharmacological differences observed between the various closely related congeners might actually be largely due to different isomeric compositions of the congeners. A more detailed discussion of the isomerism of the compounds is given in references [2,6,7,45–52]. In these papers, NMR spectroscopic and X-ray diffraction studies are also reported, the results of which strongly suggest that there is no difference between GBG and its various mono- and dialkylglyoxal analogs as isomerism is concerned and that the conventional syntheses of the compounds yield only one of the possible geometrical isomers and that this isomer is

Table 3
Repeatability of the quantitative method developed for serum samples. Recovery of bis(amidinohydrazones) from spiked human blood serum

Compound	Concentration (μg/ml)	n	Recovery (%)	Sample in water[a] (area±S.D.)	Sample in serum[a] (area±S.D.)
EMGBG	10	8	93	6.2±0.4 (±5.6)	5.8±0.2 (±3.9)
EMGBG	50	8	74	155.9±6.2 (±4.0)	115.5±4.3 (±3.7)
MGBG	10	8	108	27.3±0.7 (±2.7)	29.5±1.0 (±3.5)
MGBG	50	8	82	185.9±9.1 (±4.9)	151.1±5.9 (±3.9)

Analyses were performed by using gradient E. Recovery was calculated by comparing ultrafiltrates obtained from spiked serum to bis(amidinohydrazone) free bases dissolved in water.

[a] Values in parentheses are relative standard deviations (%).

in all cases the same, namely the *trans–trans* isomer. When the present study was initiated, it was hoped to give further evidence for or against the theory of the formation of one isomer only.

In the present study, each one of the compounds studied was invariably found to give rise to one detectable peak only and no indication of the presence of two or more isomers could be observed. The present results also indicate that even the difference between the diethyl compound DEGBG and the methylpropyl compound MPGBG is enough to make the retention times of the compounds different. Therefore, it would be expected that different isomers of a bis(amidinohydrazone) might also have different retention times. Thus, the present results are completely in line with previous NMR spectroscopic and X-ray diffraction results and do not suggest the formation of more than one isomer in conventional syntheses of the compounds. Also in MECC analyses [41,42], each bis(amidinohydrazone) studied has giving rise to one peak only, supporting the above concept.

The results obtained also indicate that the bis(amidinohydrazones) studied obviously have a distinct tendency to form ion associates with acetate ions (and probably also with other carboxylate ions) in aqueous solution. This aspect may be of biochemical significance, especially concerning the intracellular binding of the compounds. The concentration dependence of the retention times may also be of value in the elucidation of the structure of the ion associates formed.

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