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High-performance liquid chromatographic analysis of glycoamines in serum

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Abstract

This report describes the development of an HPLC-UV method for studies of glycoamines and glycoamine-like compounds in normal human serum and osteosarcoma patient serum as potential biological markers of cancer. The glycoamines, a newly recognized class of endogenous, low-molecular-mass biopolymers, are conjugates of amino acids and sugar units, containing 5 to 29 amino acid and 1 to 17 sugar units. After ultrafiltration of serum samples, reversed-phase HPLC separation with diode-array detection was used to obtain standard profiles of serum ultrafiltrates below M_r 10 000 in healthy subjects. These highly reproducible profiles utilized two-dimensional peak identification and were used to develop a statistical profile of the major glycoamine peaks in normal serum. This newly developed analytical method was subsequently used to address a key question: whether or not there is a single tumor-specific glycoamine or a family of tumor-specific glycoamines in cancer patient serum. Preliminary results suggest that this method can separate and detect glycoamines and glycoamine-like compounds in various types of cancer patient serum with a high degree of reproducibility on the basis of comparative two-dimensional identification of natural compounds and a panel of synthetic glycoamine analogs. Moreover, the method is useful for following the relative changes in the amount of a given glycoamine over an extended clinical time course. Initial results suggest that a glycoamine or glycoamine-like compound, GA-4.63, may have clinical utility in human osteosarcoma studies.

1. Introduction

The search for components of physiological fluids which may be exploited as biological markers for cancer has mainly focused on three structural categories: hormones, proteins, and nucleic acid components. Substances in blood which may indicate the presence of malignancy in humans have been the subjects of intense study (*e.g.* carcinomebryonic antigen, prostate-specific antigen, *etc.*), and the search for effective markers for cancer has utilized a number of analytical approaches.

Recently, Glinsky and co-workers [1–3] re-

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ported that a new class of endogenous substances merit interest as potential markers for cancer in humans. This class of biopolymers, glycoamines, are carbohydrate–amino acid conjugates which contain 1–17 carbohydrate and 5–29 amino acid residues. Reports have indicated that the levels of glycoamines are substantially increased in blood serum from humans and animals with various forms of solid tumors and leukemias. The elevated levels of glycoamines in the body fluids of cancer patients may be a factor that determines the malignant behavior of tumors *in vivo* [1,4–8]. Studies have indicated that the glycoamines bring about modifications in peptide–protein binding in blood plasma and subsequent alterations in the bioactivity of a wide range of protein and peptide bioregulators – neuropeptides, hormones, and peptide growth factors in malignant growth [1,9]. It has been suggested that some of the synthetic structural analogs of glycoamines may have potential as new antimetastatic and/or antiviral agents [5,7,8,10].

In 1989, Glinsky [1] reported a method for estimation of total glycoamines in serum based on the earlier work of his research group [11,12]. Glycoamines were purified from blood serum by ultrafiltration, followed by lyophilization, conventional chromatography with Sephadex G-25, and size-exclusion HPLC with Protein-Pack I-60, then final separation on gradient reversed-phase HPLC with ultraviolet absorbance detection [1]. This method was used for analysis of blood samples from more than 200 cancer patients, and Glinsky *et al.* [2,3] reported that measurement of total serum glycoamines of less than 10 kDa could detect 56–90% of certain types of human cancer in the early stages. Those results indicated that the quantitation of glycoamines in human serum has potential as a new biological marker for cancer. However, that analytical procedure was unable to resolve a key question: whether or not there is a single glycoamine that is associated with various types of cancer, or, alternatively, whether there is a family of glycoamines in serum that shows a distinct pattern for various types of human cancer.

This paper describes our studies to improve the HPLC-UV method of glycoamine quantitation in serum with emphasis on an assay that is sufficiently specific to detect the identical and structurally related glycoamine molecules in different samples with a high degree of reproducibility. Highly reproducible analyses are obviously necessary to assess the potential of glycoamines as biological markers of cancer. Two-dimensional peak identification (retention time and UV spectrum) was used, and careful selection of the maximum absorption wavelength (λ_{max}) of the major component of the HPLC peak enhances the accuracy of the quantitation. This method presents the relative changes in the amount of a given glycoamine over the clinical time course of the patient and normal subject. Observations were made on the levels of glycoamines in serum of normal subjects as well as patients with osteosarcoma, leukemia, lymphoma, melanoma and small cell carcinoma of the lung.

2. Experimental

2.1. Instrumentation

A completely automated HP-1090M HPLC system (Hewlett-Packard, Palo Alto, CA, USA) with a DR5 ternary solvent delivery system, variable-volume autoinjector, autosampler, diode-array detector, and heated column compartment was used for these studies. The liquid chromatography workstation was supported by Rev. 4.05 operation software, and consisted of an HP Model 310 computer HP-HIL 512 × 400 color monitor with bit-mapped display, and an HP-9133 H 20 mb Winchester drive with 3.5″ 710 kb micro floppy disk. A Think-Jet printer and HP 7475A plotter was used for hard copy data presentation.

2.2. Chromatographic parameters

The column used was a Vydac LC-18, 250 × 4.6 mm I.D., 5 μm, 300 Å. The column temperature was 40 ± 0.4°C. The flow-rate was 1.0 ml/min and the injection volume was 50 μl. The elution solvents employed were (A) water, HPLC grade, (B) acetonitrile–water (1/1, v/v).

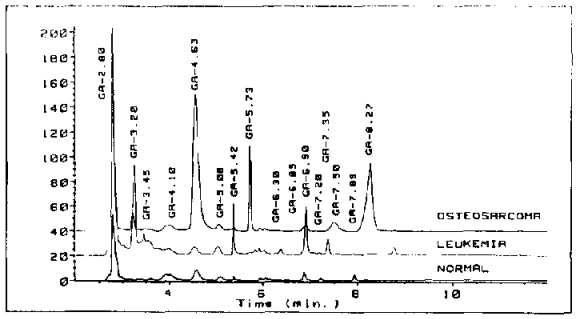


Fig. 1. RP-HPLC profiles of glycoamines and glycoamine-like compounds in sera from an osteosarcoma patient, a leukemia patient, and a normal subject. Detection at 250 nm.

The following elution solvent gradient was used from 0 to 1 min, 100% A, isocratic; 1 to 3 min, from 0% B, linearly increase to 15% B; 3 to 5 min, from 15% B, linearly increase to 50% B; 5 to 7 min, from 50% B, linearly increase to 100% B; 7 to 16 min, maintain at 100% B, isocratic. The column was equilibrated with 100% A for 15 min prior to next sample injection.

2.3. Multi-wavelength UV detection

Using the diode-array detector, selected wavelengths were used for quantitation of the glycoamines. The wavelengths selected were: 210, 225, 250, 270, 290, and 320 nm (with 4 nm band widths) and are the absorption maxima of the UV spectra of glycoamines. Figs. 1 and 2 illustrate detection to 250 and 210 nm, respectively.

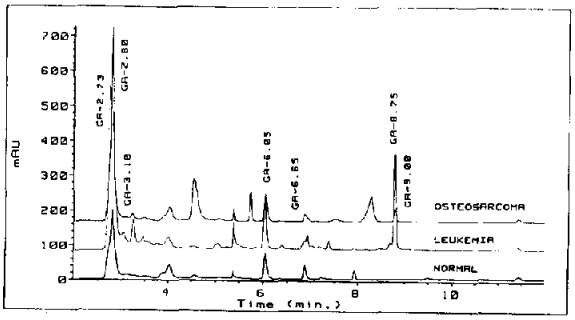


Fig. 2. RP-HPLC profiles of glycoamines and glycoamine-like compounds in sera from an osteosarcoma patient, a leukemia patient, and a normal subject. Detection at 210 nm.

2.4. Preparation of serum samples

A 100-μl volume of serum was added to 300 μl of HPLC-grade water and throughly mixed. The sample was the filtered through a 10 kDa cut-off filter (Lida Mfg. Corp., Kenosha, WI, USA) or a Millipore Ultrafree-MC 10 000 NMWL filter, and a 50-μl aliquot of the filtrate was analyzed by HPLC.

2.5. UV spectra of glycoamines

HPLC of a synthetic glycoamine, fructosyl-Trp, is shown in Fig. 3, superimposed on an HPLC separation of a serum ultrafiltrate from a cancer patient. The spectra from fructosyl-Trp and a serum glycoamine-like compound, UV 6.812, are compared in the inset in Fig. 3. The spectra of the major serum glycoamines, and glycoamine-like compounds have been obtained and have been retained in a database. Research continues on the identification of the HPLC peaks, and will include amino acid analysis, carbohydrate analysis, and mass spectrometric and nuclear magnetic resonance studies.

2.6. Quantitation of glycoamines

Due to similarities in the molecular structures of the glycoamines, some co-elution of the glycoamines was observed during HPLC separation (*e.g.* with GA-2.80, GA-3.20, GA-4.10, *etc.*). In addition, different molecules from different serum samples do elute at identical times (*e.g.* GA-3.20 from leukemia patient serum and

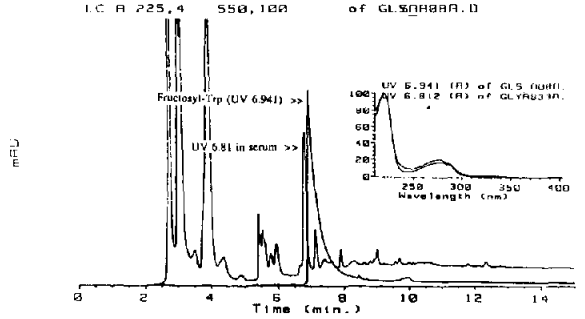


Fig. 3. RP-HPLC separation and UV spectra of the synthetic glycoamine, fructosyl-Trp (UV 6.941), and UV 6.812 in a cancer patient serum ultrafiltrate.

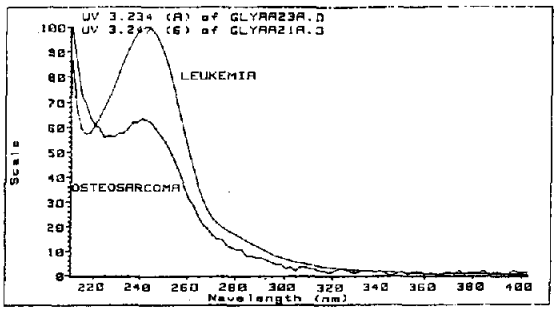


Fig. 4. Diode-array spectra of serum glycoamines (GA-3.2) from different serum samples.

osteosarcoma patient serum are different molecules, see Fig. 4). Thus, two-dimensional peak identification (retention time and UV spectra) was used, and careful selection of the maximum absorption wavelength (λ_{max}) of the major component of the HPLC peak will enhance the accuracy of the quantitation. The selected wavelength used for quantitation of each peak is listed in Table 1.

Currently we are using two external standard methods to quantitate the glycoamine peaks. The first is based on the λ_{max} of each peak, and the second is based on a non-selective wavelength, 210 nm, for all the molecules. The selected λ_{max} method gives more precise determination of the peak areas. The disagreement of the quantitation between the non-selective (210 nm) wavelength and the selected-wavelength methods may indicate co-elution of interfering molecules in the assigned glycoamine peaks.

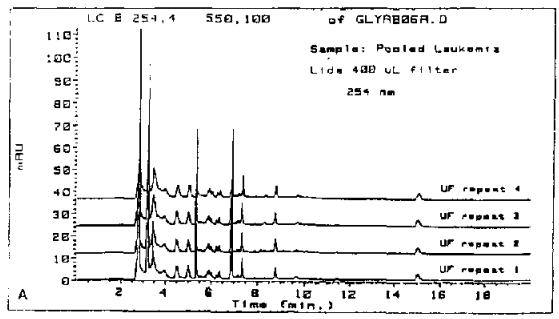
Table 1
Selected wavelength quantitation of glycoamine peaks

HPLC peak retention time (min)	Wavelength (nm)	HPLC peak retention time (min)	Wavelength (nm)
2.73	210	6.30	320
2.80	290	6.65	210
3.10	210	6.85	270
3.20	250	6.90	270
3.45	250	7.20	270
4.10	270	7.35	320
4.63	250	7.50	250
5.08	250	7.89	270
5.42	250	8.27	270
5.73	250	8.75	210
6.05	210	9.00	210

Sequential samples were collected from patients during the course of their disease and treatment, and from normal subjects. The first collected sample from a patient or normal subject (0 time) was used to calibrate the method (establish response factors). Therefore, this method yields only the relative changes of the amount of a given glycoamine over the clinical time course of the patient and normal subject.

2.7. Precision

Aliquots (400 ml) of 4× diluted pooled serum from leukemia patients were filtered through Lida 10 kDa cut-off filters and Millipore type UF filters. The HPLC chromatograms of these ultrafiltrates are presented in Figs. 5A and B. The excellent precision of the method is shown by



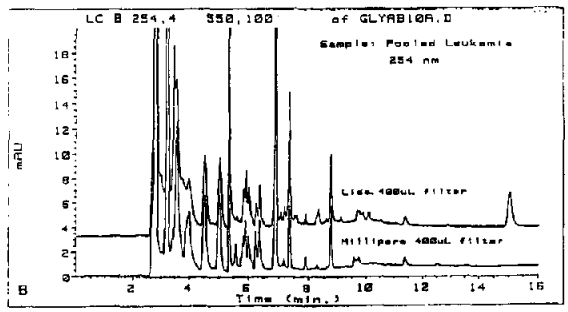


Fig. 5. (A) Four independent analyses of a pooled leukemia serum sample after filtration with Lida 10 kDa filters. (B) Comparison of serum glycoamine analysis with Millipore and Lida 10 kDa filters.

these four identical chromatograms in Fig. 5A, and the highly similar chromatograms in Fig. 5B.

2.8. Effect of ultrafilter types and serum dilution

A comparison of Millipore type UF and Lida filters revealed that the Lida filters contributed background peaks that eluted at 3–4 min and 15 min in the chromatogram. These background peaks were eliminated by washing the filters with 300 μl of water. Analyses of serum diluted ten-fold and four-fold prior to ultrafiltration showed no adverse dilution effects.

2.9. Study of the molecular size (mass) of glycoamines

Ultrafiltration of blood serum using filters with cut-offs of 10, 1, and 0.5 kDa was used to gain information on the molecular size of the glycoamines under study. Two 100 μl aliquots were taken from a cancer patient serum sample. One aliquot was filtered through a 10 kDa cut-off filter, an aliquot of that filtrate taken for analysis, then the remainder filtered through a 1 kDa cut-off filter. The second aliquot was filtered through a 0.5 kDa filter only. The data obtained from HPLC analysis of these filtrates are presented in Table 2.

The results indicate that most of the glycoamines pass through the 0.5 kDa filter, and therefore have a relatively low molecular mass. Several glycoamine peaks were smaller after filtration with the 1 kDa filter than filtration with the 0.5 kDa filter. This may have been caused by filter adsorption of glycoamines, aggregation of glycoamines after separation from serum proteins, or the co-elution of interfering components with the glycoamine peaks.

2.10. Stability of serum glycoamines

Both the glycoamine levels and profiles were found to be stable for at least 3 months during storage of serum samples at −20°C. The known factors that affect glycoamine stability are serum deproteinization and storage at temperatures above 0°C.

3. Results and discussion

Previous research has suggested that serum glycoamines merit investigation as potential biological markers for human cancer. Glinsky [1] had earlier reported an HPLC method for measuring total serum glycoamines, and this paper describes our research to advance the analysis of glycoamines in human serum.

3.1. Peak designation

UV-absorbing peaks were selected from HPLC peaks observed in normal serum, pooled serum from leukemia patients, and serum from osteosarcoma patients. Sixteen peaks were selected from chromatograms monitored at 250, 270, 290, and 320 nm (Fig. 1 and Table 1) and six peaks were selected from chromatograms monitored at 210 nm (Fig. 2). Each peak was designated as "GA-retention time in minutes" (*e.g.* GA-2.73,

Table 2
Comparison of glycoamine peaks after ultrafiltration with 10-, 1-, and 0.5-kDa filters

Filtration (cut-off, kDa)	Concentration of glycoamine found per ml serum sample at λ_{max} nm/210 nm[b]				
	GA-2.80	GA-4.63	GA-5.73	GA-6.90	GA-8.27
10	0.844/0.830	0.667/0.674	0.768/0.739	1.73/2.18	1.22/–[a]
1	0.693/0.569	0.462/0.477	0.550/0.522	1.12/1.54	1.03/–[a]
0.5	0.670/0.622	0.591/0.609	0.602/0.610	1.33/1.76	1.35/–[a]

[a] Interference.
[b] Quantities are expressed relative to a reference serum as arbitrary units, not absolute concentrations (see section 2.6).

GA-2.80, *etc.*). A total of twenty-two peaks were tentatively identified as glycoamine-like molecules (Table 1).

3.2. HPLC separation

Reversed-phase separations of the glycoamines were evaluated, and column packings with larger pore size and longer alkane chain stationary phases were found to yield better separations (*e.g.*, 300-Å pore-sized packings with C_{18} stationary phases gave better separations than packings with 60-Å pore size and C_8 stationary phases). High-purity silica is essential for the reduction of peak tailing. These observations coincide with the larger molecular mass, hydrophilic properties, and basicity of the glycoamines.

3.3. HPLC-UV of glycoamines

Two-dimensional peak identification based on simultaneous determination of retention time and diode-array UV spectra of the molecules is essential for detection and measurement of glycoamines. A comparison of chromatographic and UV spectral characteristics of synthetic glycoamine analogs with the corresponding parameters of the natural compounds of interest provides insight as to whether the synthetic compounds may be identical, structurally related or different molecules as those in serum samples. Currently our data bank contains the corresponding HPLC-UV structural analysis data for *ca.* 20 different structural analogs of glycoamines. Chromatographic behavior and UV spectra of a synthetic glycoamine analog (fructosyl-Trp) were found to be very similar to a serum glycoamine (GA-6.90) which has been found to be elevated in a number of cancer patient serum samples. However, on co-chromatography the presence of two closely eluted peaks demonstrated they were not identical. Structural analysis of the naturally-occurring glycoamines, including mass spectrometry and nuclear magnetic resonance, will be required to fully characterize this family of biomolecules.

Chromatographic retention and UV spectra of glycoamines allowed detection of structurally-related molecules having different retention times and structurally different molecules having similar retention times. Therefore, HPLC-UV analysis has the capacity to detect identical, similar and/or structurally-related molecules in different serum samples.

Seven of fourteen serum glycoamines have retention times similar to synthetic analogs (GA-2.73, GA-2.80, GA-3.20, GA-5.42, GA-6.30, GA-6.85, GA-6.90). However, only three of the seven had diode-array UV spectra identical to synthetic analogs (GA-2.73, GA-5.38, and GA-6.90). One natural compound, GA-4.10, has no synthetic analog with the same retention time, but GA-4.10 had a UV spectrum identical to GA-6.92, fructosyl-Trp, fructosyl-Tyr, and fructosyl-Phe. Fructosyl-Trp, fructosyl-Tyr, and fructosyl-Phe had retention times of 6.94, 4.72, and 6.34, respectively.

On the basis of comparisons of retention times and UV spectra of free amino acids, corresponding synthetic glycoamine analogs, and natural compounds, three glycoamine-like UV-absorbing peaks in blood serum ultrafiltrates (below 10 kDa) have been identified as Tyr (GA-4.10), Phe (GA-6.05), and Trp (GA-6.85). The UV spectra of fructosyl-Trp, -Tyr, and -Phe are identical to those of free Trp, Tyr, and Phe (data not shown). However, the retention times in the reversed-phase HPLC system employed in this study are clearly different for free amino acids and the corresponding fructosyl-amino acids: the ratio of the free amino acid retention times to the fructosyl-amino acid retention times were 0.827 for Tyr; 0.944 for Phe; and 0.969 for Trp. The co-elution experiments with the addition of corresponding standards to the blood serum ultrafiltrate and standard separation experiments supported this conclusion. The final structural confirmation of the HPLC-purified natural compounds, including mass spectrometry and nuclear magnetic resonance is required.

Therefore, two-dimensional peak identification based on simultaneous determination of retention times and diode-array spectra of the molecules of interest is essential for detection of glycoamines. Seven types of diode-array spectra

have been obtained for 16 structural analogs of glycoamines, and four of the synthetic glycoamine spectra are identical to serum glycoamine spectra. Twelve types of diode-array spectra have been obtained for 17 naturally-occurring glycoamine-like compounds in human serum, and three of the compounds have UV spectra identical to three of the synthetic analogs studied to date.

Future HPLC research on the glycoamines will focus on ion-exchange HPLC separations, as ion-exchange is expected to yield improved separations of glycoamines which have similar molecular structures but slightly different charge distributions. With regard to HPLC detection, electrochemical detection (ED) of glycoamines will be investigated. Comparisons of reversed-phase and ion-exchange retention characteristics, and comparisons of UV and ED responses are expected to provide useful structural information on the glycoamines prior to actual structural studies.

3.4. HPLC analysis of glycoamines in normal subject and cancer patient serum

To date, five glycoamine-like peaks are found to be present in quantifiable amounts in cancer patient serum while present in only trace amounts in normal serum samples. Several glycoamine peaks, which represent major peaks in cancer patient serum, are either absent or at trace levels in serum from normal subjects, raising the possibility of these molecules as cancer-specific markers.

HPLC profiles of cancer patient serum and serum from normal subjects were compared, and significant differences were observed (Figs. 1, 6 and 7). The major observations were:

(1) Significant differences in the amounts of GA-2.80 were observed between serum from cancer patients and normal subjects (Figs 1, 6, and 7). At least three minor components are known to co-elute with GA-2.80, but the increase in peak size observed in the cancer patient samples as compared to the normal samples is due to the major glycoamine component, GA-2.80.

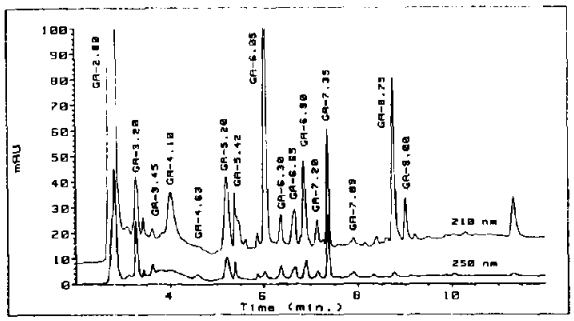


Fig. 6. HPLC-UV analysis of glycoamines in leukemia patient serum.

(2) Glycoamine GA-3.20 is observed in cancer patient serum but not in normal subject serum. Although a molecule in leukemia patient serum elutes in the same area as GA-3.20, spectral data reveal it is not the same molecule as observed in the osteosarcoma patient sample. We have designated these molecules as: GA-3.20-O (osteosarcoma) and GA-3.20-L (leukemia).

(3) Five glycoamines are found to be present in cancer patient serum, but absent or in trace quantities in normal subject serum: GA-3.20-O, GA-4.63, GA-5.73, GA-7.50, and GA-8.27.

3.5. HPLC of glycoamines – implications for osteosarcoma

The value of this approach to investigating potential biological markers may be illustrated by recent, initial results from studies on osteosarcoma. In this study, HPLC-UV analyses of 17

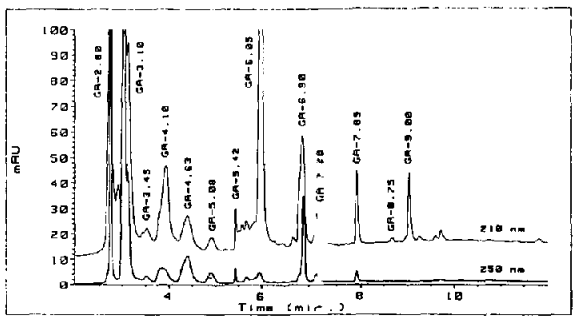


Fig. 7. HPLC-UV analysis of glycoamines in small cell lung carcinoma patient.

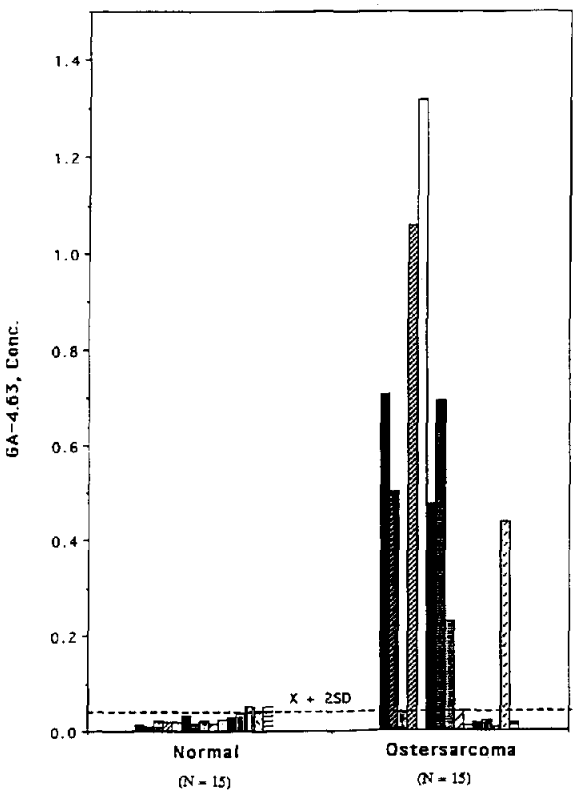


Fig. 8. Serum levels of GA-4.63 in normal subjects and osteosarcoma patients.

individual glycoamine-like compounds were conducted on pretreatment serum samples from 16 patients with osteosarcoma, and the results compared to analyses of 20 normal subjects. Seventeen glycoamine-like molecules were studied in osteosarcoma patient serum at presentation and during the course of anti-neoplastic therapy.

Statistically significant changes in serum glycoamine levels were observed in all 16 osteosarcoma patients, in particular the level of GA-4.63. It was also observed that multiple changes in the concentrations and patterns of glycoamines were characteristic for human osteosarcoma. In other studies we found no corresponding magnitudes of GA-4.63 for patients with leukemias, lymphomas, small cell lung carcinomas, and melanomas. Only two of 16 osteosarcoma patients had normal serum levels of GA-4.63 (Fig. 8). A report will follow on serum glycoamine levels in sequential samples from patients with osteosarcoma and on the full structural characterization of GA-4.63. This glycoamine-like compound may possess diagnostic potential and possibly prognostic correlations with human osteosarcoma.

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