Journal of Chromatography, 224 (1981) 381—388
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 890

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY QUANTITATION OF N-ACETYLNEURAMINIC ACID IN MALIGNANT MELANOMA AND BREAST CARCINOMA

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(First received January 5th, 1981; revised manuscript received March 5th, 1981)

SUMMARY

A rapid isocratic high-performance liquid chromatography (HPLC) method for quantitation of serum N-acetylneuraminic acid (NANA) is described. Separation is achieved on an Aminex HPX-87 cation-exchange resin using a 0.006 *N* sulfuric acid mobile phase. Compared with the more conventional thiobarbituric acid (TBA) method, HPLC is more reliable and has a much improved maximum sensitivity of 0.8 nmol/ml. In a limited study of malignant melanoma patients' sera, HPLC gave slightly higher values for NANA than TBA. In a more detailed study of breast carcinoma patients with measurable tumour burden, HPLC and TBA methods were used on the same sera and compared with concurrent carcinoembryonic antigen (CEA) determinations. HPLC resulted in a clear improvement in discrimination between tumour burden groups compared with either the TBA method or CEA.

INTRODUCTION

We have been interested in the clinical significance of sialoglycoproteins in

0378-4347/81/0000—0000/$02.50

cancer patients' sera. Our studies have shown that serum sialoglycoprotein elevations, as reflected by total serum sialic acid (N-acetylneuraminic acid, NANA), occur in association with a variety of neoplasms and that these elevations are related to tumour burden and prognosis [1—3]. This suggests that total serum NANA can be a valuable clinical tumour marker. In these studies NANA was measured by the thiobarbituric acid (TBA) method [2], essentially as described by Warren [4]. While this method is relatively inexpensive and easy to perform, there may be problems with interfering substances [4], the interassay variation is suboptimal [2], and the basic procedure is relatively time consuming.

The several methods devised for quantitation of sialic acids vary in complexity, sensitivity and specificity. We have examined high-performance liquid chromatography (HPLC) as a method of NANA measurement. In addition to reliability, sensitivity and selectivity, we wished to determine if the relative simplicity of HPLC would lend itself to use as a clinical NANA assay. In this study we describe an HPLC assay for NANA, compare this with the TBA method [2], and illustrate how HPLC may be used for clinical measurement of NANA in cancer patients' sera.

MATERIALS AND METHODS

Serum collection

Whole blood was collected in 10-ml glass vacutainer tubes and the samples allowed to clot at room temperature for 1 h. After centrifugation at 500 *g* for 10 min, the sera were removed, placed in polypropylene tubes and stored at —70°C until used. Prior to assay, the serum samples were allowed to thaw at room temperature. The reliability of this procedure has been previously examined by us [2].

TBA assay

NANA was hydrolyzed from serum sialoglycoproteins by incubation for 1 h at 80°C in a mixture of 0.1 ml test serum with 2 ml 0.1 *N* sulfuric acid. The hydrolysate was then cooled for 5 min in a 20°C water bath. Further analysis was essentially as described by Warren [4], but with the modifications as described by us previously [2]. As previously reported by us, the intra-assay variation (± 2 S.D.) was 1.9% and the interassay variation was 10%.

HPLC

Sialoglycoprotein hydrolysis was performed as for the TBA assay, except that 0.23 μmol/ml N-acetylglucosamine (NAGA) (Sigma, St. Louis, MO, U.S.A.) was incorporated as an internal standard. NANA was analyzed by cation exclusion chromatography on a Model 7000B chromatograph (Micromeritics, Norcross, GA, U.S.A.) using an Aminex HPX-87 strong cation-exchange resin column designed for organic acid analysis, 300 × 7.8 mm at 42°C (Bio-Rad Laboratories, Richmond, CA, U.S.A.). A mobile phase of 0.006 *N* sulfuric acid was used at a flow-rate of 0.65 ml/min and a pressure of $7 \cdot 10^6$ N m^{-2}. The column effluent was monitored by a UV detector at 206 nm and 0.05 absorbance span, using an LKB 2138 Uvicord S detector fitted with an

HPLC micro flow-cell and the photodiode output recorded at a chart speed of 0.33 cm/min. The injection volume for each sample was 50 μl and the total run time per serum sample was 20 min.

NANA content of test samples was calculated from peak height ratios using the NAGA described above as an internal standard and referring to a standard curve using concentrations of NANA (Sigma) from 0.32 to 6.5 μmol/ml in 0.1 *N* sulfuric acid.

Mass spectrometry

Following injection of NANA standard and serum hydrolysate, column effluent was collected at 0.5-min intervals and fractions representing UV absorbance peaks were pooled and lyophilized.

The lyophilized material was placed in a Reacti-Vial (Pierce, Rockford, IL, U.S.A.) and 25 μl of N,O-bis-trimethylsilyl-trifluoroacetamide (Pierce) added. The stoppered tube was then heated in a heating block until the silylation reaction was complete as indicated by total solubilization of the substances [5, 6]. Trimethylsilyl ether-ester derivatives were immediately subjected to high-resolution mass spectrometric analysis on a Finnigan Model 4000 mass spectrometer. The 70-eV electron impact ionization fragmentation patterns were interpreted according to the mass spectra described by Kamerling and Vliegenthart [7].

Stability studies

NANA standards, NAGA standards and serum samples were heated in 0.1 *N* sulfuric acid at 80°C for 1, 5 and 24 h. Standards and serum in distilled water were incubated in parallel for comparison. Additional hydrolyzed serum samples were analyzed immediately and compared with the same hydrolysate analyzed after maintenance at 4°C for 24 and 48 h.

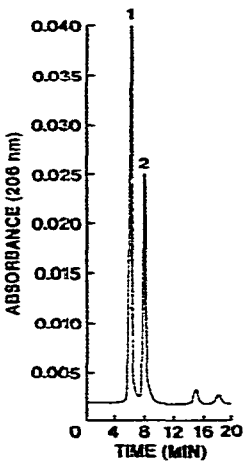
Patients

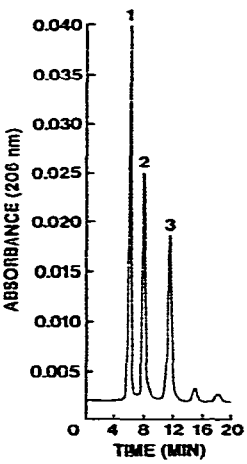
Forty malignant melanoma patients were selected, representing a broad range of serum NANA concentrations as determined by the TBA method. Fifty-eight breast carcinoma patients were selected for study on the basis of objective measurable tumour burden and assigned to tumour burden groups much as we have previously described [2]. Group I included 20 patients with no evidene of residual neoplasm between 4 and 8 weeks after surgical excision of all known carcinoma; group II, 18 patients, had limited recurrent disease confined to the chest wall and estimated at less than 5 g; and group III consisted of 20 patients known to have more advanced regional or distant metastatic disease estimated at more than 5 g. Breast carcinoma patients had plasma carcinoembryonic antigen (CEA) determined by the Roche method (Roche Laboratories, Nutley, NJ, U.S.A.) on samples obtained at the same time as serum for sialic acid. In addition to specimens from cancer patients, 40 normal control sera were selected from our serum bank for comparison. These were from 40 females with an age range of 19—91 year. All sera were analyzed for sialic acid by both the TBA and HPLC methods as described above. Thirty of these control sera were also available for CEA testing.

RESULTS

HPLC of NANA

The elution of a standard NANA solution from the Aminex HPX-87 column with retention time of 7.8 min is identical to that of the second UV absorbance peak of the serum hydrolysate (Fig. 1). The eluate representing this peak was collected and the trimethylsilyl derivative analyzed by mass spectrometry. Characteristic derivative fragmentions of 668, 624, 478, and 298 daltons identified NANA [7].





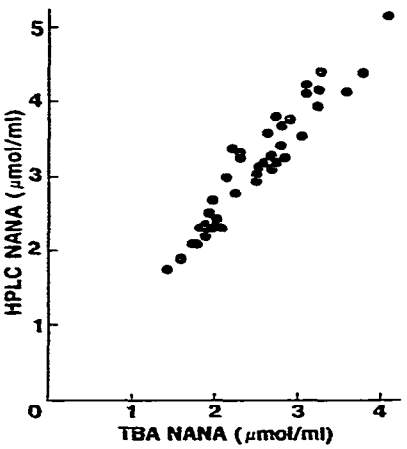


Fig. 1. Chromatogram of serum hydrolysate. Retention time of NANA (peak 2) = 7.8 min. 1 = Acid injection peak.

Fig. 2. Chromatogram of serum hydrolysate with added 0.23 μmol/ml NAGA as internal standard. Retention time of NAGA (peak 3) = 11.36 min. 2 = NANA. 1 = Acid injection peak.

Fig. 3. Linear regression analysis of TBA and HPLC methods for NANA in malignant melanoma patients. Correlation coefficient = 0.98.

The chromatogram of NAGA added to serum hydrolysate is displayed in Fig. 2. NAGA elutes as a sharp peak at 11.36 min, independent of other eluted material. This suggests NAGA could serve as an internal standard provided it remained stable under assay conditions. The effect of heat and acid were examined by HPLC after incubation of serum and standard NANA and NAGA solutions in 0.1 *N* sulfuric acid for 1, 5 and 24 h. The loss for NAGA was acceptable with a reduction of 2.5% in the first hour, decreasing to 1.7% per hour in the final observation period. The reduction in NANA during the first hour was 9.5% in keeping with results of others [8, 9]; decreases in the final observation period were 2.8% per hour. Storage of hydrolysate or standard solutions of NANA or NAGA at 4°C after the standard 1 h hydrolysis at 80°C did not cause any further loss up to 48 h.

NANA concentration was linearly related to peak height, and this relationship was maintained even at low NANA concentrations. The maximum sensitivity for NANA in our system was 0.8 nmol/ml with a signal-to-noise ratio > 3.

On the basis of the above results the HPLC assay detailed in Materials and Methods was used for the analysis of serum samples. Intra-assay variation on 20 serum analyses was 2.1 ± 2% S.D. and the interassay variation on 15 analyses was 5.2%.

Serum studies

HPLC and TBA assays were directly compared in 40 malignant melanoma patients. In each case, both assays were performed on the same serum sample. As shown in Fig. 3, the concordance was very high (correlation coefficient = 0.98). This illustration also suggests HPLC tended to give slightly higher values for NANA than the TBA method. Fig. 4 shows this more clearly and demonstrates that the difference is significantly greater at higher NANA concentrations ($F = 16.04$ with 2 and 77 degrees of freedom, $p < 0.001$). In this analysis the normal control sera were included to provide NANA values in the lower range.

NANA results for each tumour burden group of the 58 breast carcinoma patients are shown in Fig. 5. Again, generally higher values are apparent for

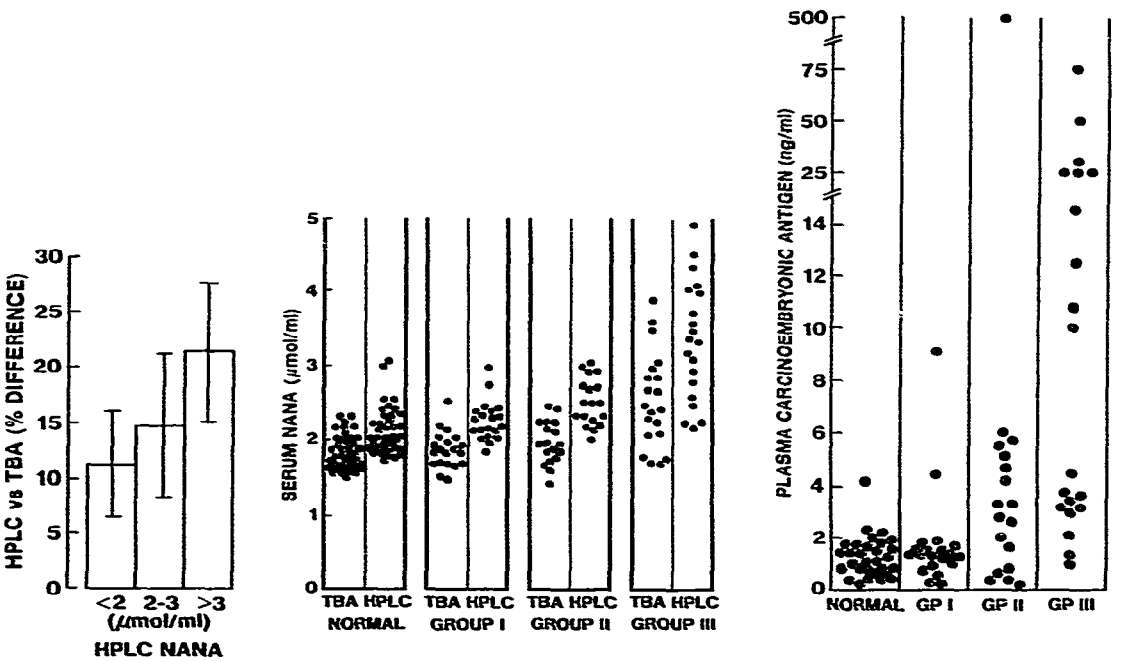


Fig. 4. Relationship of TBA and HPLC methods. Ordinate: percent difference between HPLC and TBA: $\left(\frac{\text{HPLC NANA} - \text{TBA NANA}}{\text{HPLC NANA}}\right) \times 100$. Abscissa: three ranges of HPLC NANA values.

Fig. 5. NANA serum concentrations in normals and breast carcinoma patients determined by both TBA and HPLC on the same sera.

Fig. 6. CEA concentrations concurrently determined on the same breast carcinoma patients represented in Fig. 5. Thirty concurrent normal control plasma were available for CEA testing.

the HPLC method. Results of pairwise statistical analyses by the Mann—Whitney test are displayed in Table I. While there was significant discrimination between groups for HPLC in each case, TBA results did not attain statistical significance for groups I vs. normal, I vs. II, and II vs. normal. Of the remaining three comparisons, confidence levels (p values) were more remarkable for HPLC in each case.

TABLE I

BREAST CARCINOMA GROUP COMPARISONS (MANN—WHITNEY TEST) FOR NANA BY HPLC, NANA BY TBA METHOD, AND CEA

Groups compared	Significance (p)		
	HPLC	TBA	CEA
I vs. normal	0.02	NS*	NS**
I vs. II	0.006	NS	0.02
II vs. III	0.0005	0.002	0.01
II vs. normal	0.00007	NS	0.004**
III vs. I	0.000004	0.0002	0.000009
III vs. normal	0.00000002	0.000005	0.0000001**

*NS, not significant, $p > 0.05$.
**Normal control group included 30 sera.

Concurrent plasma CEA values were available for all breast carcinoma patients and 30 of 40 normal controls (Fig. 6). Pairwise statistical analyses (Table I) appeared marginally more significant for CEA than TBA NANA, but in each case confidence levels were more remarkable for HPLC NANA than CEA.

DISCUSSION

Methods for detection and quantitation of sialic acids have relied principally on colorimetric procedures using reagents such as resorcinol [10], diphenylamine [11], dimethylaminobenzaldehyde [12], and thiobarbituric acid [4, 13]. Most of these procedures are relatively insensitive and lack specificity. Although fluorimetry [8, 14] offers a relatively simple means of improving sensitivity, interfering substances pose a problem [15]. Specificity can be greatly improved through additional preliminary purification steps [15—18], or, to a lesser extent, by development of empirical mathematical formulas to control for interfering substances [4]. Other assays have improved specificity, but more elaborate laboratory procedures impose additional practical limitation. The enzymatic assay of Brunetti et al. [19] requires purified N-acylneuraminic acid aldolase. The ion-exchange method of Svennerholm [17] is time consuming, and both gas—liquid chromatography and mass spectrometry require substrate conversion to volatile derivatives [6, 7, 20, 21].

In contrast to colorimetric methods, HPLC offers the potential of improved sensitivity and selectivity and the advantage of ease of operation compared with the more elaborate procedures.

In our study the HPLC assay was compared directly with the TBA method. In general, HPLC is more easily performed and more reliable. The interassay variation of 5.2% was almost half that of the TBA method [2]. A combination of factors may be responsible for the improved reliability of HPLC analysis: the assay is more direct, NAGA is used as an internal standard, and mathematical manipulations for controlling interfering substances are eliminated. HPLC was 250-fold more sensitive. The lower limit of sensitivity of 0.8 nmol/ml is comparable to more elaborate sensitive chromatographic techniques [22—24]. Fluorimetry can be more sensitive, but there are associated problems with specificity. Although we have not demonstrated absolute specificity for NANA using HPLC, we have not detected any interfering substances, and this is supported by our mass spectrometric studies.

Comparative serum studies have shown general concordance between HPLC and TBA methods (Fig. 3). The source of limited but consistent higher NANA results by HPLC may be related to as yet unidentified effects of serum on the Aminex HPX-87 column during HPLC. The discrepancy between assays is perhaps better explained by serum interference in the TBA assay [25—27]. For example, L-fucose, known to be elevated in breast cancer patients such as ours [28, 29], inhibits color formation in the TBA assay as do many other serum components [30].

Serum NANA elevations have previously been reported in breast carcinoma patients. In a limited study Macbeth and Bekesi [31] reported that NANA elevations in breast carcinoma were confined to advanced disease patients. As part of a larger study Mrochek et al. [23] found a correlation between serial NANA levels and response to treatment in 21 unstaged patients. Hogan-Ryan et al. [32] have recently reported on serum NANA levels in 19 advanced disease patients and 46 patients sampled after primary surgery when there would presumably have been no evidence of residual disease. While significance levels are not reported, those with advanced disease clearly had higher serum NANA concentrations. In a comparison with CEA, the authors felt that NANA was possibly a better tumour marker. Since we studied a different selected population and used a different CEA test, our results are not strictly comparable to those of Hogan-Ryan et al. [32]. However, it is of interest that our results for NANA measured by TBA are in keeping with those reported by others.

Of greater importance, our breast carcinoma study illustrates the potential advantage of HPLC for serum NANA quantitation. Pairwise statistical analysis demonstrated a clear improvement in discrimination among tumour burden groups for HPLC NANA compared with either TBA NANA or CEA. The apparent HPLC sensitivity for relatively small tumour burden is also in contrast to our previous patient studies using the TBA method [2, 33—35]. Since the same sera were used for both HPLC and TBA measurement, the improved discrimination of HPLC in this study is best explained by the superior intra- and interassay reliability.

In our hands the simplicity and reliability of HPLC quantitation of NANA make it a valuable laboratory tool with ready application for clinical use.

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