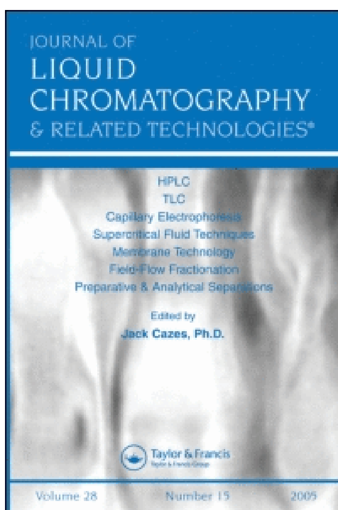
This article was downloaded by:
On: *25 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination and Analysis of Human Serum Alpha-1-acid Glycoprotein by Liquid Chromatographic Methods

T. Kremmer[a]; M. Boldizsár[a]; J. Kovács[a]; E. Paulik[a]; K. Bencsik[a]; B. Szajáni[b]
[a] Department of Biochemistry, National Institute of Oncology H-1122 Budapest, Hungary [b] Reanal Factory of Laboratory Chemicals, Budapest, Hungary

To cite this Article Kremmer, T. , Boldizsár, M. , Kovács, J. , Paulik, E. , Bencsik, K. and Szajáni, B.(1995) 'Determination and Analysis of Human Serum Alpha-1-acid Glycoprotein by Liquid Chromatographic Methods', Journal of Liquid Chromatography & Related Technologies, 18: 6, 1207 — 1218

To link to this Article: DOI: 10.1080/10826079508009285

URL: http://dx.doi.org/10.1080/10826079508009285

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(6), 1207-1218 (1995)

DETERMINATION AND ANALYSIS OF HUMAN SERUM ALPHA-1-ACID GLYCOPROTEIN BY LIQUID CHROMATOGRAPHIC METHODS

T. KREMMER[1], M. BOLDIZSÁR[1], J. KOVÁCS[1], E. PAULIK[1], K. BENCSIK[1], AND B. SZAJÁNI[2]

[1]*Department of Biochemistry*
National Institute of Oncology
H-1122 Budapest, Hungary
[2]*Reanal Factory of Laboratory Chemicals*
H-1147 Budapest, Hungary

Downloaded At: 07:37 25 January 2011

ABSTRACT

Human serum alpha-l-acid glycoprotein (orosomucoid, AGP) with particular interest as a tumor marker was measured, separated, purified and analyzed for its carbohydrate constituents. In order to demonstrate the changes in the concentration and composition of serum AGP induced by malignant diseases an improved method of sample preparation based on solvent extraction, ion exchange and reversed phase liquid chromatographic methods were applied. In preliminary studies significantly elevated serum AGP levels and increased fucose content in the carbohydrate moiety were found in cancer patients in comparison to the healthy individuals.

INTRODUCTION

Alpha-l-acid glycoprotein (orosomucoid, AGP) is a characteristic and dominant fraction of human serum

Copyright © 1995 by Marcel Dekker, Inc.

sialoglycoproteins with a molecular mass of 40 KD, an unusually high carbohydrate content (45%), and a large number of sialyl residues (1, 2). Changes in the serum AGP concentration are considered as a marker of several metabolic disorders. Highly elevated plasma AGP levels were found in patients with inflammatory diseases and cancer (3-7). Recently, we demonstrated that sialic acid measured in a tumor marker was originated primarily from the plasma AGP content (8). It has been shown that plasma AGP is polymorph and individual molecular variants with slightly different chemical composition, electrophoretic mobility and affinity to lectins can be distinguished (5, 9-11). It has also been reported that the compositional changes in human serum AGP induced by various acute phase conditions are in coincidence with the alterations in the antennary structure of glycan chains (10, 12-14). It is of particular interest, therefore, to investigate the anomalies in the molecular heterogeneity of serum AGP induced by malignant diseases. Recent methods for measuring and preparation of human serum AGP are based on either immunological (diffusion-precipitation, affinity) or salting-out and ion exchnage chromatographic techniques (1, 3-6, 10-13). These procedures may have various difficulties in the time, expenses and purity of the product, or in the selectivity of quantitation (15-16). In the present paper we worked out a single step sample preparation and a microanalytical ion exchange chromatographic method for measuring the AGP content of human serum in cancer patients as well as in healthy individuals. In order to determine the changes in the sialic acid and sugar constituents, serum AGP was prepared and purified by ion exchange chromatography and analyzed by reversed phase HPLC methods.

Downloaded At: 07:37 25 January 2011

MATERIALS

Chemicals and reagents used were of analytical grade from E. Merck (Darmstadt, Germany) unless stated otherwise. All solvents were of HPLC quality (LiChrosolv, Merck). Prepacked columns and Q-Sepharose Fast Flow were purchased from the producers. Bis-tris-propane, reference monosaccharides (glucose, galactose, mannose, fucose), N-acetyl neuraminic (sialic) acid, serum protein fractions including human serum alpha-1-acid glycoprotein and alpha-1-antitrypsin were obtained from Sigma Co. (St Louis, MO.).

Apparatus

The Pharmacia-LKB FPLC System composed of two P-500 pumps, an LCC-500 Liquid Chromatographic Controller and a UV-1 Monitor (at 280 nm) was applied to the ion exchange and gel chromatographic (desalting) separations using MonoQ HR 5/5 (5x0.5 cm I.D., 10 μm), Q-Sepharose Fast Flow (30x2.54 cm I.D., 45-165 μm) and Fast Desalting HR 10/10 (10x1 cm I.D.) columns (Pharmacia-LKB, Uppsala, Sweden). Measuring of sialic acid and neutral sugars was carried out on a LiChrosorb RP-18 column (25x0.46 cm I.D., 5 μm) with a Merck-Hitachi L-6200A Intelligent Pump and a D-2500 Chromato-Integrator equipped with an L-4250 UV-VIS and a Shimadzu RF-530 fluorscence detector. HPLC columns were thermostated at 30°C.

METHODS

Sample preparation

Downloaded At: 07:37 25 January 2011

Venous blood was drawn from healthy volunteers and hospitalized cancer patients after overnight fasting. Blood was allowed to clot and serum was separated by centrifugation (1000g, 4°C, 10 min), and kept at -20°C until use. One vol of human serum was diluted with 3.5 vol of ice-cold water and extracted with 15 vol of chloroform-methanol mixture (2:1 v/v) by vigorous shaking at 0°C for 45 min. Solvent phases were separated by centrifugation (1000g, 4°C, 20 min) and the methanol-water upper phase (extract) was collected and evaporated 10-20 fold in vacuum for the preparative ion exchange chromatography.

Determination of the serum AGP content

In the microanalytical procedure 100 μl of human serum was treated according to the sample preparation with proportionally measured solvent volumes in a glass stoppered centrifuge tube (8x1.5 cm I.D.). After centrifugation 500 μl of upper phase was applied directly to the MonoQ HR 5/5 column. Separation of AGP was performed using a combined pH/NaCl gradient elution system (17) with solvents: A - 50 mM bis-tris-propane/HCl buffer (pH:7.5) containing 10% (v/v) of methanol, and

B - 50 mM bis-tris-propane/HCl buffer (pH:9.5) containing 350 mM NaCl according to the elution program: A for 2.5 ml, B 0-100% for 20 ml, B 100% for 2.5 ml and A for 5 ml (equilibration). For measuring the serum AGP content the MonoQ HR 5/5 column was calibrated with commercial AGP in a range of 10-200 µg/500 µl of sample volume. Serum AGP concentration was calculated from the linear correlation of ug AGP vs peak height (AUFS 0.1) values obtained considering that 500 µl of methanol-water extract was equivalent to 65 µl of serum. Results were expressed as mg AGP/dl of serum.

Preparative isolation of human serum AGP

In the preparative scale separation of human serum AGP 1-2 ml concentrates of the methanol-water extract were applied to a Q-Sepharose Fast Flow column (30x2.54 cm I.D.) and eluted with proportionally increased buffer volumes excepted that methanol was omitted from the buffer A. The gradient elution program was as follows: A for 75 ml, B 0-100% for 525 ml, B 100% for 150 ml, then equilibration with 300 ml of buffer A. Flow rate was 2 ml/min and 20 ml fractions were collected with detection at 280 nm. Eluates were desalted on a Fast Desalting HR 10/10 column eqilibrated with distilled water, lyophilized and analyzed by polyacrylamide gel electrophoresis as described earlier (8). Protein content was determined according to Hartree (18).

Reversed phase HPLC methods

N-Acetyl neuraminic (sialic) acid content of AGP samples was determined by the method of Hara et al (19). 1,2-Diamino-4,5-methylenedioxybenzene (DMB) derivative of sialic acid was separated by isocratic elution with methanol-acetonitrile-water (25:4:91) as mobile phase. Fluorescence detection was at excitation: 373 nm, and emission: 448 nm. For quantitative measuring calibration curve was plotted with commercial sialic acid. The major monosaccharides (galactose, mannose, fucose) were measured according to Eggert and Jones (20). Dansyl monosaccharides were separated on a LiChrosorb RP-18 column by isocratic elution with acetonitrile-water (1:4 v/v) containing 10 mM formic acid, 40 mM acetic acid and 1 mM triethylamine. Flow rate was 1.0 ml/min and detection at 254 nm. Quantitation was done by means of reference monosaccharides.

Downloaded At: 07:37 25 January 2011

RESULTS

In the present work a representative sialoglycoprotein AGP was separated and purified partially using a single-step solvent extraction with chloroform-methanol. Chromatographic and electrophoretic analysis of the sample preparation demonstrated that under the conditions reported here solvent extraction resulted in the precipitation and bulk elimination of major plasma proteins (mostly albumin, alpha-1-antitrypsin, globulins, Figure 1), while serum AGP remained solved in the methanol-water (upper) phase, probably, due to its exceptionally acidic and soluble character (8). The relative enrichment of AGP in the methanol-water extract and an improved technique of ion-exchange chromatography allowed either the quantitative and selective measuring of serum AGP content (Figure 1-B), and/or the scaling-up for its preparative isolation (Figure 2). As regards as the analytical method studies on pooled human serum samples supplemented with AGP in a range of 10-500 mg/dl showed a recovery of 96% (3.1% C.V.). Accuracy of the method was 2.2% (C.V.) in average in a range of 10-200 mg AGP/dl of serum, and sensitivity was found to be 5 μg AGP in 500 μl of sample (AUFS 0.1). Serum concentrations and retention time of AGP could be reproduced with 95% (1.14% C.V.). Our results concerning the serum total AGP content obtained for healthy individuals as well as for cancer patients (Table I) are in accordance with the figures of others (5, 10, 22). One of the major advantage of the sample preparation is that the methanol-water extract of human sera can directly be applied to the MonoQ HR 5/5 column in a volume of 500 μl. It has been observed that using 10% (v/v) of an organic modifier (methanol, acetonitrile) in the mobile phase (buffer A) during the equilibration and gradient elution reduced the retention of some contaminants (e.g. nucleotides, peak 2 in Figure 1-B) by 15-20%, and resulted in sharper peaks and better resolution. It has to be noted, on the other hand, that the methanol content in the mobile phase suppressed the UV absorption of proteins by 30-40% and, therefore, the sensitivity of detection. Figure 2 shows the ion exchange chromatographic separation of human serum AGP on a Q-Sepharose Fast Flow column. In contrast to the polymer based MonoQ particles the methanol content of the sample should be removed when the polysaccharide Q-Sepharose packing was used. Selectivity of the preparative scale isolation was checked by polyacrylamide gel electrophoresis and serum AGP (fractions

Downloaded At: 07:37 25 January 2011

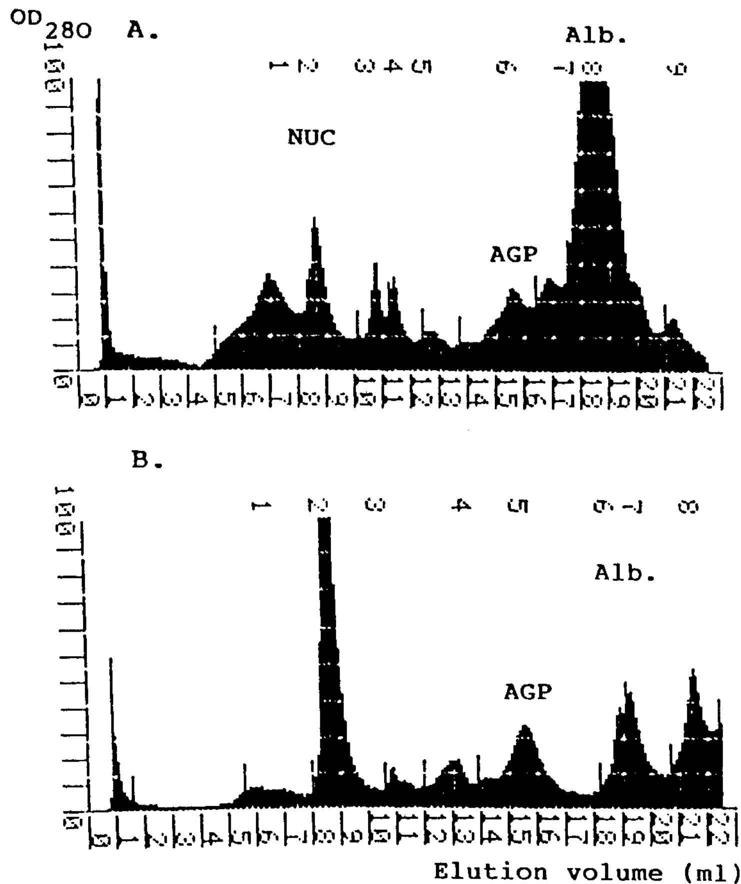


FIGURE 1.

Ion exchange chromatography of human serum proteins on a MonoQ HR 5/5 column (5x0.5 cm I.D., 10 µm) operated by the Pharmacia-LKB FPLC System. Gradient elution see Methods. Flow rate: 0.5 ml/min. Detection: 280 nm (AUFS 0.1). Abbreviations: NUC - Nucleotides, AGP - Alpha-1-acid glycoprotein, Alb - Albumin.

A - Separation of the total serum protein content, (Sample: 50 µl of diluted (1:25) human serum)

B - Separation of proteins in the methanol-water extract of human serum (Sample: 500 µl of extract).

Downloaded At: 07:37 25 January 2011

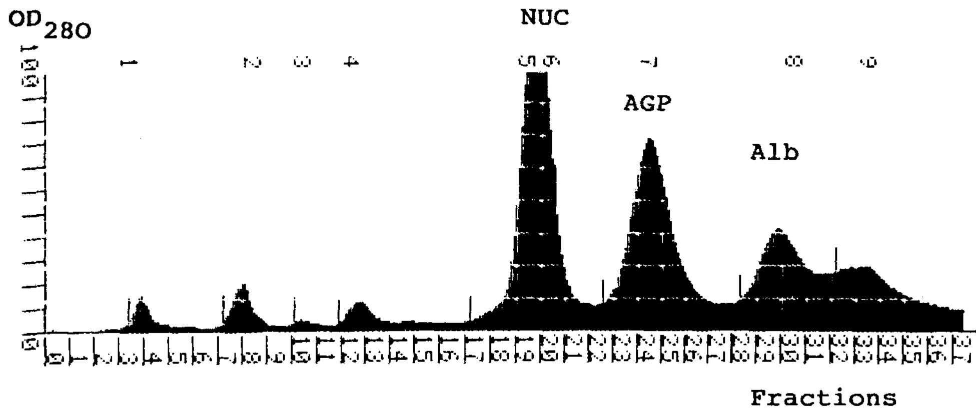


FIGURE 2.

Preparative ion exchange chromatographic separation of human serum AGP on a Q-Sepharose Fast Flow column (30x 2.54 cm I.D., 45-165 µm). Gradient elution see Methods. Flow rate: 2.0 ml/min. Detection: 280 nm (AUFS 0.2-0.5). Sample: 2.0 ml concentrated (10x) methanol-water extract of human serum. Fractions: 20 ml. Abbreviations see Figure 1.

Downloaded At: 07:37 25 January 2011

24-25, Figure 2) could be obtained in a purity over 99%. Alpha-1-antitrypsin contamination could not be detected.

Analytical data concerning the sialic acid and monosaccharide constituents of human serum AGP are summarized in Table I. Similarly to others (3-7) we have found significantly higher total AGP levels in the sera of cancer patients (mainly in ovary, colon and breast carcinomas) in comparison to the healthy individuals. However, there was no specificity for the various forms of neoplasma. Our investigations demonstrated some characteristic compositional changes of serum AGP in the presence of advanced malignancy. Figure 3 shows that the distribution of neutral sugars in serum AGP was similar to that of reported for the healthy individuals (19). On contrary, the non-protein constituents (mainly neutral sugars) seemed to be markedly increased, and significantly higher fucose content was measured in the serum AGP of cancer patients supporting the relevance of serum fucose determination as a marker in malignant diseases (21-23).

TABLE 1
Human Serum AGP Content and Constituents in Healthy Individuals and Cancer Patients

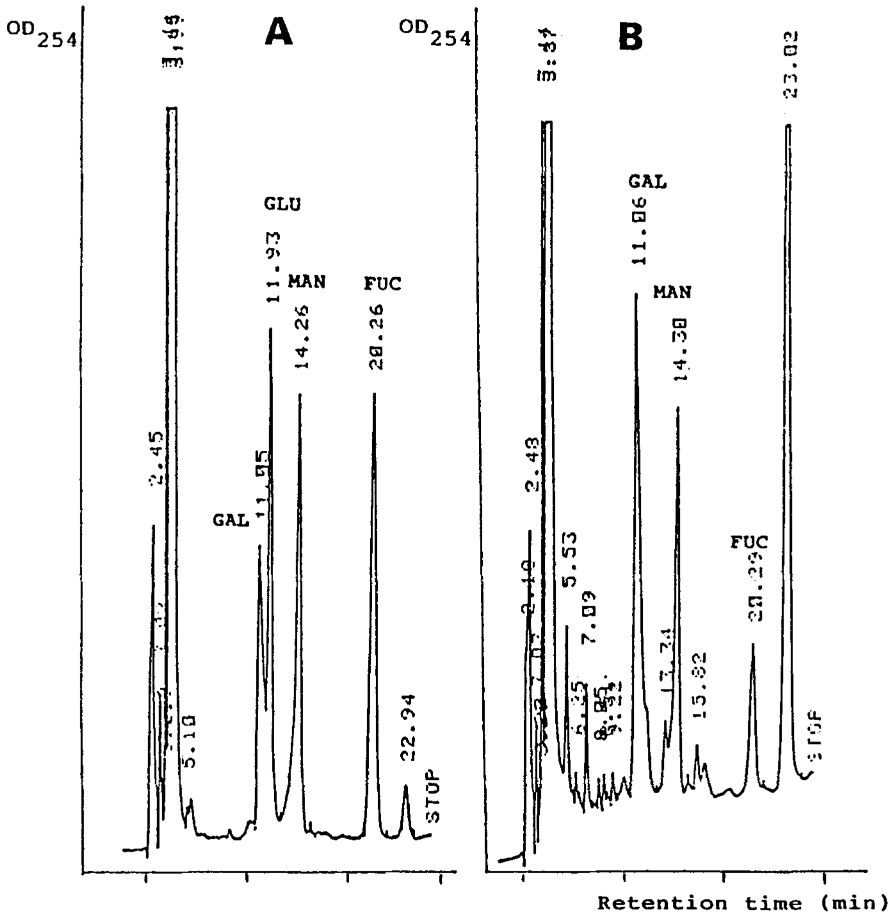
Serum concentrations mg/dl	Healthy individuals (n=9)	Cancer patients * (n=15)
Total AGP	83.3 ± 21.5 (62-104)	255 ± 62 (193-317)
Protein content	47.0 ± 0.9	130.6 ± 3.5
Sialic acid	9.7 ± 1.7	26.4 ± 2.4
Galactose	17.6 ± 2.3	61.3 ± 6.3
Mannose	8.3 ± 1.4	32.1 ± 4.3
Fucose	0.7 ± 0.2	4.6 ± 0.5

* Ovary, colon and breast carcinomas

DISCUSSION

Sialoglycoproteins, in general, comprise a large variety of natural compounds with various molecular characteristics and biological activity, and as carrier biopolymers represent the typical structural and functional forms of carbohydrate and sialic acid transport in the circulation (1, 16). Isolation of these solutes from a complex matrix with particular respect to the preservation of their structural integrity and function requires a reliable combination of sample preparation and separation techniques. The present method of sample preparation based on solvent extraction and combined with an improved technique of ion exchange chromatography proved to be suitable either for analytical purposes in measuring the serum AGP content, or for the preparative scale purification of AGP. In comparison to other methods providing high capacity (Cohn's fractionation), or selectivity (immunoaffinity) in the isolation of AGP, the solvent extraction seems to be faster and less expensive. While the automatized techniques (RIA, Delphia) are not comparable, but in price, the selectivity and accuracy of the analytical ion exchange chromatography prevails the immunodiffusion or precipitation techniques in the serial investigation of individual serum samples. From the results presented here it can be concluded that some disadvantages of the

Downloaded At: 07:37 25 January 2011



Downloaded At: 07:37 25 January 2011

FIGURE 3.

HPLC separation of dansyl monosaccharides on a LiChrosorb RP-18 column (25x0.46 cm I.D., 5 µm). Isocratic elution with acetonitrile-water (1:4 v/v) containing 10 mM formic acid, 40 mM acetic acid and 1 mM triethylamine. Flow rate: 1 ml/min. Detection: 254 nm.
A - Refernce monosaccharides: GAL - Galactose, GLU - Glucose, MAN - Mannose, FUC - Fucose.
B - Monosaccharides of human serum AGP.

former methods having difficulties in the separation and purification of AGP from alpha-1-antitrypsin (15) can be overcome by selecting a suitable sample preparation, an appropriate buffer system (bis-tris-propane) with a simultaneous pH-NaCl gradient elution program, and a macroporous anion exchanger with $-CH_2-N^+(CH_3)_3$ functional groups (MonoQ, Q-Sepharose). Hydroxylapatite proved to be useful for the elimination of the alpha-1-antitrypsin contamination (15), but without the ability of fractionating a complex protein mixture. The more favourable hydrodynamic properties and the protective effects of the polysaccharide matrix prefer to the use of dextran-based ion exchangers (Q-Sepharose).

Investigating the compositional changes in human serum AGP induced by the malignant diseases our results seem to confirm that AGP represents one of the main structural forms of serum fucose content elevated in cancer, and probably, molecular variants of AGP different from the normally existing biopolymers appear and increase in the blood of cancer patients. To clarify the molecular bases behind the changes of sugar constituents related to the malignant diseases further attempts are needed to investigate the polymorphism of AGP.

Downloaded At: 07:37 25 January 2011

ACKNOWLEDGEMENTS

The authors thank Mrs Vera Scheuring, Mrs Gyöngyi Márványos, Mrs Beata Akacs and Andrea Napholcz for the valuable technical assistance. We are indebted the DOJINDO Laboratories (Kumamoto, Japan) for the generous supply of DMB. This work was supported by the Hungarian Research Fund (OTKA T-4375), and the Ministry of Welfare (ETT T-412).

REFERENCES

1. K. Schmid, "α_1-Acid Glycoprotein", in The Plasma Proteins, F. W. Putnam, ed., Academic Press, New York, 1975, pp. 184-228.

2. T. Takagi, "Determination of protein molecular weight by gel permeation chromatography equipped with low angle laser scattering photometer", in Progress in HPLC, Vol. 1. Gel Permeation and Ion-exchange Chromatography of Proteins and Peptides, H. Parvez, Y. Kato, S. Parvez, eds., VNU Science Press, Utrecht, 1985, pp. 27-41.

3. P.A. Ganz, M. Baras, P. Y. Ma, R. M. Elashoff, Cancer Res., 44: 5415-5421 (1984)

4. P. A. Ganz, P. Y. Ma, H. J. Wang, R. M. Elashoff, J. Clin. Oncol., 5: 472-479 (1987)

5. J. S. Hansen, V. A. Larsen, T. C. Bog-Hansen, Clin. Chim. Acta, 138: 41-47 (1984)

6. C. B. Eap, J. F. Fischer, P. Baumann, Clin. Chim. Acta, 203: 379-386 (1991)

7. J. Mittermuller, B. Weidinger, Electrophoresis, 13: 785-786 (1992)

8. S. Végh, T. Kremmer, M. Boldizsár, K. A. Gesztesi, B. Szajáni, Clin. Chim. Acta, 203: 259-268 (1991)

9. J. S. Hansen, T. C. Bog-Hansen, B. Pedersen, K. Neland, Electrophoresis, 10: 574-578 (1989)

10. S. K. Moule, M. Peak, S. Thompson, S. A. Turner, Clin. Chim. Acta, 166: 177-185 (1987)

11. J. Breborowicz, A. Mackiewicz, Elelctrophoresis, 10: 568-573 (1989)

12. D. Biou, C. Bauvy, H. N'Guyen, P. Codogno, G. Durand, M. Aubery, Clin. Chim. Acta, 204: 1-12 (1991)

13. M. J. Treuheit, C. F. Costello, H. B. Halsall, Biochem. J., 283: 105-112 (1992)

14. M. J. Treuheit, H. B. Halsall, Chromatographia, 35: 90-92 (1993)

15. Y. Funde, S. Wada, S. Imaoka, S. Hirotsune, M. Tominaga, S. Tanaka, T. Kishimoto, J. Chromatogr., 381: 149-152 (1986)

16. J. M. H. Kremer, J. Wilting, L. M. H. Janssen, Pharmacol. Rev., 40: 1-47 (1988)

17. H. Lindblom, U.-B. Axiö-Frederiksson, E. H. Cooper, R. Turner, J. Chromatogr., 273: 107-116 (1983)

18. E. F. Hartree, Anal. Biochem., 48: 422-427 (1972)

19. H. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura, Y. Ohkura, Anal. Biochem., 164: 138-145 (1987)

Downloaded At: 07:37 25 January 2011

20. F. M. Eggert, M. Jones, J. Chromatogr., 333: 123-131 (1985)

21. J. E. Mrochek, S. R. Dinamore, D. C. Tormey, T. P. Waalkes, Clin. Chem., 22: 1516-1521 (1976)

22. G. A. Turner, A. W. Skillen, P. Busmah, D. Guthrie, J. Welsh, J. Harrison, A. Kovalski, J. Clin. Pathol., 38: 588-592 (1985)

23. P. S. Patel, B. R. Baxi, S. G. Adhvaryn, B. B. Balar, Anticancer Res., 10: 1071-1074 (1990)

Received: August 30, 1994
Accepted: September 29, 1994

Downloaded At: 07:37 25 January 2011