Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1027 (2004) 161–166
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Analysis of Tamm–Horsfall protein by high-performance liquid chromatography with native fluorescence

Zak K. Shihabi [a,*], Mark E. Hinsdale [a], Anthonny J. Bleyer [b]

[a] *Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA*
[b] *Department of Nephrology, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA*

Abstract

Tamm–Horsfall (TH) is a large glycoprotein which originates in the kidney and is very abundant in the urine. This protein has been measured mainly by immunoassays. Here we describe a different approach for its measurement based on high-performance liquid chromatography (HPLC) using a molecular exclusion column with native fluorescence detection in the ultraviolet range. This method in addition to measuring the level of the protein has the advantage of detecting changes in size or aggregation. Urine, 1 ml was mixed with 100 μl of 30% NaCl and left at 37 °C for 30 min. The urine was centrifuged at 12 000 rpm for 20 s. The precipitate was vortex-mixed and dissolved in a triethanolamine buffer. A 20 μl aliquot was injected on a Macrosphere GPC column which was eluted with phosphate buffer and the effluent was detected by a fluorometer set at 280 nm for excitation and 325 nm for emission. Since the protein has a very large molecular mass compared to other urinary and serum proteins we did not experience any interference. It elutes as the first peak (in ∼2.5 min on a 500 Å and 2.7 min on 1000 Å). The protein precipitates rapidly <60 min at 37 °C. The detection in the UV is sensitive for this protein down to 1 mg/l in absence of any concentration steps. The method was linear between 1 and 100 mg/l. The R.S.D. was 10.4% (mean 62, $n = 10$). The mean level in 42 normal individuals was 31 mg/g creatinine and in 30 patients with proteinuria (different renal disorders) was 23 mg/g creatinine.

Keywords: Tamm–Horsfall protein; Proteins; Glycoproteins; Uromodulin

1. Introduction

Tamm–Horsfall (TH) is an abundant urinary glycoprotein originating in the thick ascending limb of the loop of Henle cells of the kidney. It is a glycosylphosphatidylinositol (GPI)-anchored protein and it is the most abundant protein in normal urine. Originally it was described by Morner early in 1895 (see [1]) as mucoprotein and later isolated and characterized by Tamm and Horsfall [2] in 1950 after precipitation with 0.58 M NaCl. Independently, uromodulin (UM) was isolated in 1985 from the urine of pregnant women and was found to have the same amino structure of TH; however, the carbohydrate content especially the mannose chains and the antigenity is slightly different.

The monomeric form of this protein (under reducing conditions) has a molecular mass of ca. 80 000–930 000; however, it is usually present in very large aggregates of $M_r > 5 \times 10^6$. It has 639 amino acids and about 48 cysteine residues. Four separate homologous domains of TH exhibit similarity to that of the epidermal growth factor. It contains about 30% carbohydrates [3,4] which are essential for its inhibition of viral hemagglutination and also important for its binding of certain cytokines (IL-1, IL-2, TNF) [1]. It tends to gel especially in the presence of calcium, sodium ions, albumin and radiocontrast media. The zona pellucida (ZP) domain present in this protein is responsible for its polymerization [5].

Since the work of Tamm and Horsfall several investigators attempted to define its function. Its gel property is important for water permeability of the epithelial cells of the thick ascending limb of Henle [6]. It is also thought to act as an adhesion molecule and to be involved in stone formation where it is present in the core of the stones. It may be involved in defense against infection since it binds to uropathogens such as *E. coli* and viruses [2,7]. It binds a number of proteins, including those on surfaces of bacteria and viruses [7]. It inactivates enzymes of those organisms, possibly by reacting with ionic cofactors, such as divalent metals [8]. Ionic binding of monovalent cations (e.g. Na^+) and repulsion of anions, such as Cl^-, suggest a role for it

* Corresponding author. Tel.: +1-336-716-2639; fax: +1-336-716-9944.
E-mail address: zshihabi@wfubmc.edu (Z.K. Shihabi).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.09.008

in renal salt and water balance. It is thought also that it regulates or modulates the circulating level of some cytokines too.

Based on DNA analysis recently we found, that mutations in the uromodulin gene involving cysteine substitutions occur in medullary cystic kidney disease and familial juvenile hyperuricaemic nephropathy disorders which are characterized by juvenile onset of hyperuricaemia, gout, and progressive renal failure [9]. These mutations probably lead to an alteration in the tertiary structure of this protein. Both disorders are associated with interstitial pathological changes resulting in fibrosis. In other words, TH protein is vital for the normal function of the kidney.

This protein has been measured mainly by different immunoassays (enzyme-linked immunosorbent assay, ELISA) methods [10–13]. These methods require special equipment and the use of anti-sera which is not easily available. Here we describe a different approach for TH protein measurement based on HPLC using a molecular exclusion column with native fluorescence detection operating in the ultraviolet range. This method has the advantages of detection size or aggregation changes of this molecule. The ELISA detects certain epitope sites on the molecule while the described HPLC detect molecular mass. Thus, the two methods can be considered complementary for the study of TH protein especially for the altered or the pathologic protein.

2. Materials and methods

2.1. Quantification procedure

For quantification, 1 ml urine was mixed with 100 μl of 30% NaCl and left at 37 °C for 30 min to precipitate. The urine was centrifuged at 12 000 × *g* for 20 s. The supernatant was discarded thoroughly. The precipitate was washed gently with 1000 μl of 2% NaCl without disturbing it and finally was dissolved (vortex-mixed for 20 s) in 1 ml of a buffer of triethanolamine 15 mmol/l, pH 7.5 containing EDTA, 4 mmol/l. A 20 μl aliquot of the solution was injected on the column.

2.2. Protein aggregation studies

For study of the aggregations of TH protein a through wash of the precipitate is important to remove the small molecular mass interferences. Sodium chloride, 3 g was dissolved in 100 ml of urine, left in at 4 °C for 4 h and the precipitate was washed thoroughly three times with 1 ml of 2% saline and finally dialyzed for 4 h against water at 4 °C [2].

2.3. HPLC

Two columns, Macrosphere GPC 500 and 1000, 7 μm, 250 mm × 4.6 mm i.d. (Alltech Associates, Deerfield, IL, USA) were tried. Both eluted with phosphate buffer, 7 mmol/l, pH 5.7 at flow rate of 0.7 ml/min and the effluent was detected with a model RF-10AXL Fluorometer (Shimadzu Instruments, Colombia, MD, USA) set at 280 nm for excitation and 325 nm for emission.

2.4. Dialysis

For comparison to the quantification method by precipitation, 200 μl of urine was placed in dialysis membrane (8000 molecular weight) and dialyzed against the triethanolamine buffer for 4 h. Samples were stirred and mixed well before injection of a 20 μl aliquot on the column.

2.5. Standard

TH protein was precipitated from pooled urine as in Section 2.2, dissolved in water, re-precipitated and finally dialyzed against the triethanolamine buffer. The isolated TM was dried on filter paper and standards were prepared from that.

3. Results and discussion

Tamm–Horsfall protein unfortunately is not that water soluble [14,15] especially after precipitation or when present in a concentrated solution. It is not detected easily by agarose gel electrophoresis because of the solubility problem. The use of native fluorescence detection in this study offers the needed high sensitivity to avoid the problem of solubility associated with concentrated solutions or the extra steps needed for protein staining. The molecular exclusion column of 500 Å is suitable for studying proteins of molecular mass of $20\,000 - 7.5 \times 10^6$. When isolated fresh, TH elutes rapidly in this column as the first peak, in ∼2.3 min reflecting its high molecular size [16] (Fig. 1, top). Using another column of 1000 Å which is suited for molecular mass of $30\,000 - 25 \times 10^6$ the peak elutes as the first peak also at ∼2.7 min. This indicates indirectly that the molecular mass of the TH aggregates is $7000 - 20 \times 10^6$ The 1000 Å column gave sharper and taller peaks (better sensitivity). In other words both columns can be used for the analysis.

Urine can be injected directly except for the presence of the many native fluorescent compounds which overwhelm the detector and the numerous polar compounds which adhere to the packing material and over time can ruin the expensive column. However, dialysis can decrease this problem especially the interferences from the small molecules but not the large ones (Fig. 1, middle). The dialysis has the advantage of avoiding the precipitation and dissolution steps; in other words it avoids the possibility of denaturation from precipitation (Fig. 1, bottom), but it is not quite suitable for routine use.

TH is a very large protein present in large aggregates of $M_r > 5 \times 10^6$ [16]. It is the largest protein in urine compared to other urinary or serum proteins. It is almost 100

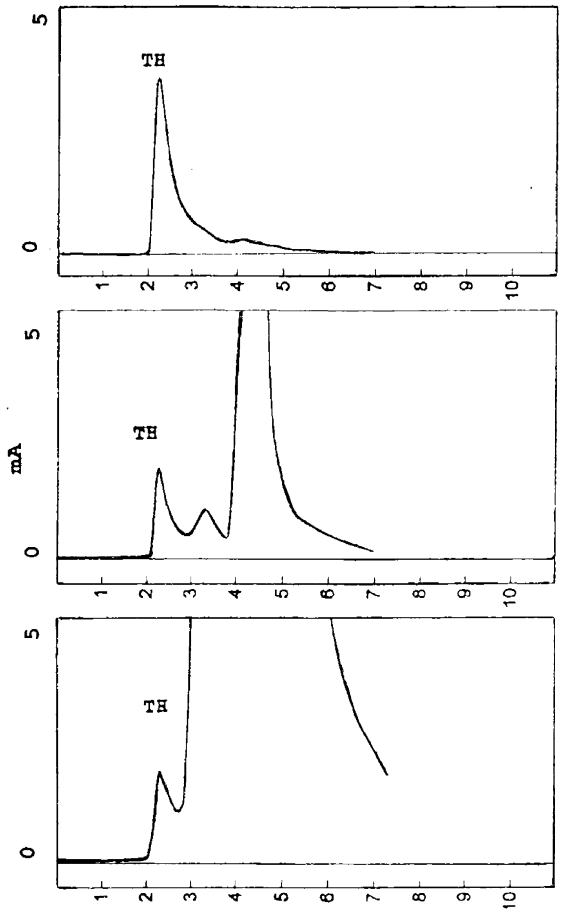

Fig. 1. Analysis of: (top) a standard (100 mg/l), (middle) a patient by precipitation and (bottom) the same patient by dialysis (*x*-axis, time in minutes).

times larger than the gamma globulins which occasionally present in urine. Because of this high molecular mass we did not experience any interference on this type of columns, the 500 and 1000 Å. Furthermore, the reference values obtained by this method (Table 1) are similar to those by other methods as will be described later. Addition of serum to urine samples did not affect the quantification of TH protein. Thus, the protein can be precipitated for quantification once without a need for extra cleanup steps. An extra wash step removed some of the low-molecular-mass interferences yielding cleaner chromatograms but without improving the quantification results. Protein precipitation from pregnant women gave one peak at the same void volume of TH.

Table 1
Tamm–Horsfall protein level in different groups

Group	Mean		
	n	Cr (mg/g)	S.D.
Normal	42	31.6	29.6
Proteinuria	30	23.6	23.9
Pregnancy	9	49.5	29.6

TH protein can undergo changes in size or aggregation. The aggregates are possibly important in pathological states. As shown recently, this protein is rich in cysteine which can undergo mutation [9]. These mutations can alter the aggregation because of the tendency of the SH group for binding or oxidation. Two types of changes in the aggregates are observed using this column for TH, (S1) and (S2). The S2 aggregates are smaller in size than S1, Fig. 2. Standards, over time, tend to break or to desegregate slowly to form fragments (S1) eluting after the main peak at ∼2.8 min (Fig. 2). The change in size increases with time, Fig. 3 and is accelerated at higher temperatures. Since cysteine residues are important for the function of this protein we studied the effect of added cysteine and related compounds on the aggregates. In presence of 0.2% of cysteine, *N*-acetylcysteine, glutathione or Cleland's reagent different amounts of small aggregates (S2) appear at ∼3.9 min (Fig. 2, bottom) which

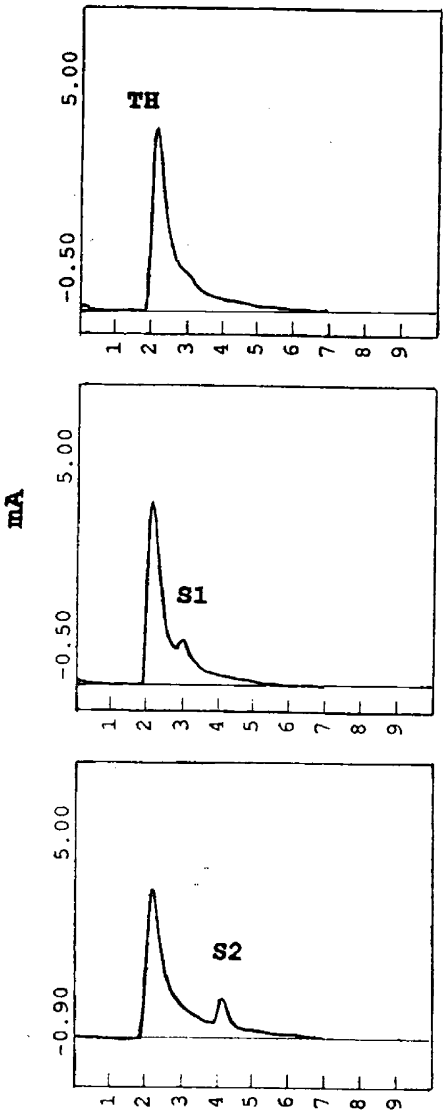

Fig. 2. Detection of TH protein smaller size aggregates S1 and S2 (*x*-axis, time in minutes).

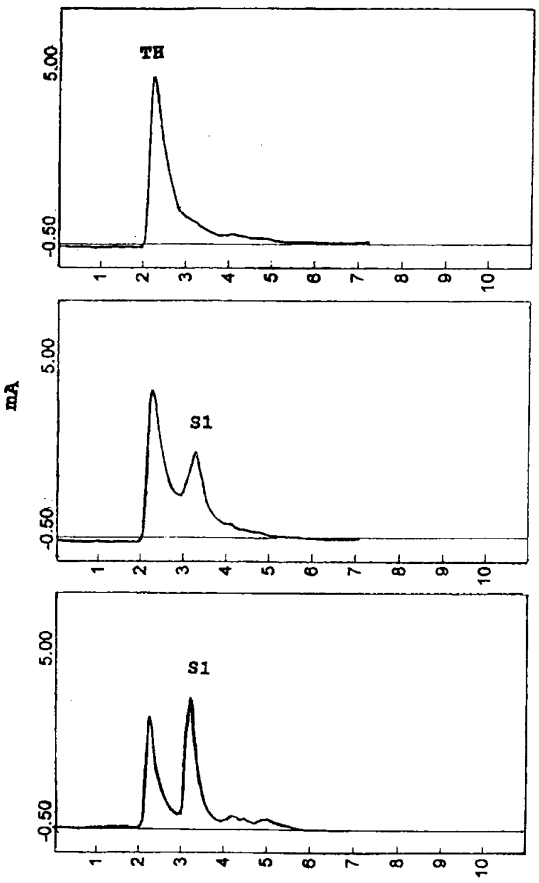

Fig. 3. Break down of the standard with storage: (top) fresh, (middle) 10 days and (bottom) 30 days later at 4 °C (*x*-axis, time in minutes).

increase with time. Cystine was much slower in producing this aggregate. Sodium chloride and $CaCl_2$ decrease the solubility of TH but also cause the formation of S2. Furthermore, as the salt increases from 0 to 3%, the solubility of the large aggregate decreases too; thus the ratio of small to large aggregates seems to increase with increases in the salt concentration. This conversion is partially reversible and in agreement with the work of Kobayashi and Fukuoka [14] who found that with increase in sodium ion concentration especially over 100 mmol/l TH decreases in solubility. The importance of this aggregation in the different mutation of this protein is under investigation.

TH protein traditionally has been precipitated by bringing the NaCl concentration to 0.58 mol/l [2]. The optimum concentration of NaCl for protein quantification by this method as illustrated in Fig. 4 is between 20 and 40 g/l very close to what has been used [2]. Interestingly small but variable amounts of TH protein precipitated without the need for addition of salt. This is mainly because urine contains variable amounts of sodium ions depending on the diet. This points out to the importance of analysis of fresh urine without long term storage. The protein starts to precipitate rapidly in a few minutes after addition of the salt (Fig. 5). Unexpectedly, it precipitated much better at 37 °C when compared to 4 °C (Fig. 6). Since precipitation can cause a loss in the recovery of this protein, or loss through denaturation we compared this method to simple dialysis. The correlation between the two procedures is good (dialysis $= 0.85 \times$ precipitation $+$ 1.29, $r = 0.99$, $n = 19$). The precipitation was much faster, gave cleaner chromatograms and was more suited for routine work than that by dialysis. In the dialysis method, occasionally the TH protein precipitated on the membranes. Other proteins tended to clog the pores. The addition of extra water was necessary in order to dissolve these proteins.

The detection of TH by the native fluorescence is very convenient and sensitive for this protein down to 1 mg/l in absence of any concentration or staining steps. It is also easy to dissolve the sample in less volume in order to increase the sensitivity. However, we did not find any clinical need for that. The method was linear between 1 and 100 mg/l with the minimum detection level (3× baseline noise of

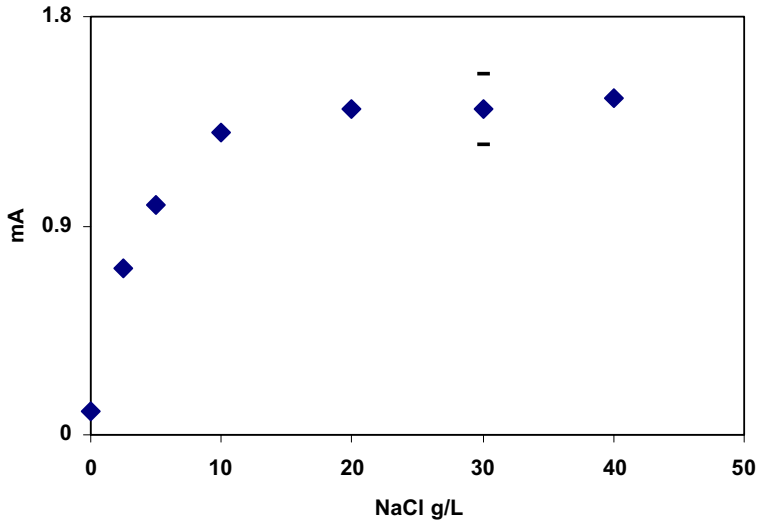

Fig. 4. Optimum concentration of NaCl (final concentration) for Tamm–Horsfall protein precipitation.

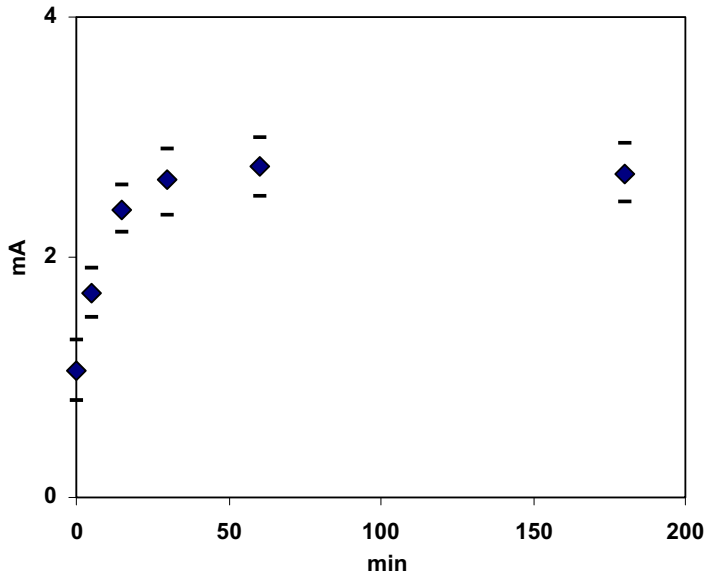

Fig. 5. Precipitation of Tamm–Horsfall protein after addition of the s 34 g/l alt at 37 °C at different time periods.

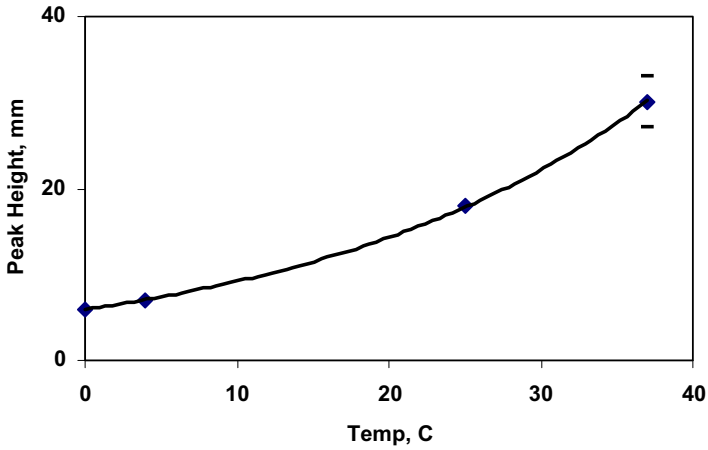

Fig. 6. Precipitation of Tamm–Horsfall protein after addition of the salt 34 g/l at different temperatures (4 h incubation).

0.9 mg/l). The R.S.D. was 10.4% (mean 62, $n = 10$). Because the concentration as mg/l of different compounds in urine is quite dependant on the urine flow and the degree of hydration, expressing the compounds as a ratio to creatinine is a common method in routine urinary clinical analysis to overcome these variables [17]. Based on this fact, the mean level in 42 normal individuals was 31 mg/g creatinine, in 30 patients with proteinuria (different renal disorders) the level was 23 mg/g creatinine and in nine pregnant females the level was 49.5 mg/g creatinine (Table 1). The average for TH found in the normal individuals is similar to what has been reported earlier [1].

4. Conclusions

The described HPLC method for TH protein analysis based on molecular size and native fluorescence is simple, sensitive enough for clinical use and offers information complementary to that obtained by the immunoassays. In addition to measuring the level of TH protein it is suitable for studying the factors, which affect its molecular size or aggregation.

References

[1] S. Kumar, A. Muchmore, Kidney Int. 37 (1990) 1395.
[2] I. Tamm, F.L. Horsfall, Proc. Soc. Exp. Biol. Med. 74 (1950) 108.
[3] A.P. Fletcher, A. Neuberger, W.A. Ratcliffe, Biochem. J. 120 (1970) 425.
[4] A.P. Fletcher, A. Neuberger, W.A. Ratcliffe, Biochem. J. 120 (1970) 417.
[5] L. Jovine, H. Qi, Z. Williams, E. Litscher, P.M. Wassarman, Nat. Cell. Biol. (2002) 457.
[6] F.D. Menozzi, A.S. Debrie, J.P. Tissier, C. Locht, K. Pethe, D. Raze, Microbiology 148 (2002) 1193.

[7] L.A. Hawthorn, A.W. Bruce, G. Reid, Urol. Res. 19 (1991) 301.
[8] R.E. Wenk, B.S. Bhagavan, J. Rudert, Pathobiol. Annu. 11 (1981) 229.
[9] T.C. Hart, M.C. Gorry, P.S. Hart, A.S. Woodard, Z. Shihabi, J. Sandhu, B. Shirts, L. Xu, H. Zhu, M.M. Barmada, A.J. Bleyer, J. Med. Genet. 39 (2002) 882.
[10] M.G. Dehne, J. Boldt, D. Heise, A. Sablotzki, G. Hempelmann, Anaesthesist 44 (1995) 545.
[11] T. Yamamoto, H. Miyata, T. Fujiyama, T. Kinoshita, S. Maki, Nephron. 59 (1991) 440.
[12] H.H. Reinhart, N. Obedeanu, D. Walz, J.D. Sobel, Am. J. Clin. Pathol. 92 (1989) 199.
[13] J.S. Hunt, R.J. Peach, M.C. Brunisholz, K.L. Lynn, A.R. McGiven, Immunol. Methods 91 (1986) 35.
[14] K. Kobayashi, S. Fukuoka, Arch. Biochem. Biophys. 388 (2001) 113.
[15] J. Wieslander, P. Bygren, D. Heinegard, Clin. Chim. Acta 78 (1977) 391.
[16] F.J. Schweigert, J. Raila, S. Haebel, Vet. Res. 33 (2002) 299.
[17] Z.K. Shihabi, R.P. Schwartz, M.J. Pugia, Ann. Clin. Lab. Sci. 31 (2001) 99.