OTOANALİZÖRE UYGULANABİLİR SERUMDA DOYMAMIŞ DEMİR-BAĞLAMA KAPASİTESİ ÖLÇÜMÜ: DİREKT BİR METOT

AN AUTOMATED PROCEDURE FOR ASSAY OF SERUM UNSATURATED IRON-BINDING CAPACITY: A DIRECT METHOD

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Özet

Bu çalışmada, otoanalizöre uygulanabilir direkt bir metot serumda doymamış demir bağlama kapasitesinin ölçümü için kullanıldı. Serum örneklerini manuel olarak önceden herhangi bir işleme tabi tutmadan, satürasyon ve reaksiyon basamakları otoanalizör tarafından yapıldı. Bu maksatla, doymamış demir bağlama reaktifi ferrous amonyum sülfat ile karıştırıldı ve R1 (reaktif 1) pozisyonuna yerleştirildi. Renk reaktifi R2 (reaktif 2) pozisyonuna bırakıldı. Otoanalizörü proğramladıktan sonra, alet numuneleri küvete alıp R1 ile karıştırıldı ve R2 nin ilavesine kadar satürasyona devam etti. R2 ilavesini takiben, fazla bağlanmamış demir ölçüldü. Metot iki farklı analizöre uygulandı (Mitsubishi SZ 818 ve Hitachi 717). Bu metotla manuel metot arasında iyi bir korelasyon elde edildi ($r=0.955$, $p<0.001$). Sonuç olarak, serumda doymamış demir bağlama kapasitesinin güvenilir tesbitinin açık sistemli, selektif ve diskret bir çok otoanalizörde papılabileceği kanaatine varıldı.

Anahtar kelimeler: *Serum demiri, Ansatüre demir-bağlama kapasitesi.*

Summary

In this study, an automated direct method was introduced for the determination of serum unsaturated iron-binding capacity. Without any pretreatment of serum sample with iron manually, the saturation and the reaction steps were carried out by the autoanalyzer. For this purpose, unsaturated iron-binding reagent was mixed with ferrous ammonium sulfate and placed in R1 (reagent 1) position. The colour reagent was placed in R2 (reagent 2) position. After programming the analyzer, it sampled the serum into the cuvette, mixed with R1 and lasted the mixture for saturation until the addition of R2. Following R2 addition, the excess, unbound iron was measured. The method was applied to two different autoanalyzers: Mitsubishi SZ 818 and Hitachi 717. A good correlation was obtained with manual method ($r=0.955$, $p<0.001$). The conclusion is that a simple, reliable determination of serum unsaturated iron-binding capacity can be made in many autoanalyzers which are open system, discrete, and selective.

Key words: *Serum iron, Unbounded iron-binding capacity.*

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Introdution

Alterations in the level of serum iron and iron-binding capacity have been observed in a number of conditions. In most instances, it is not the value of either one, but the values of both the iron and the iron binding capacity, that is of most clinical significance. It is clear that the reliable methods are required in order to determine serum iron and iron binding capacity.Most of iron in serum is bound to protein, mainly to transferrin, which is a plasma iron transport protein and binds ferric (Fe^{+3}) ions in plasma pH. Each molecule of transferrin combines with two ferric ions. However, only a portion of the transferrin molecule is saturated with iron (about 25-30 %) (1). The unsaturated iron binding capacity (UIBC) denotes the available iron-binding sites of transferrin present in serum. On the other hand, the amount of iron that serum transferrin can bind when completely saturated is the total iron-binding capacity (TIBC). The TIBC can be defined as serum iron plus UIBC (2). In present study an automted, direct method was introduced for iron binding capacity determination.

Materials and Methods

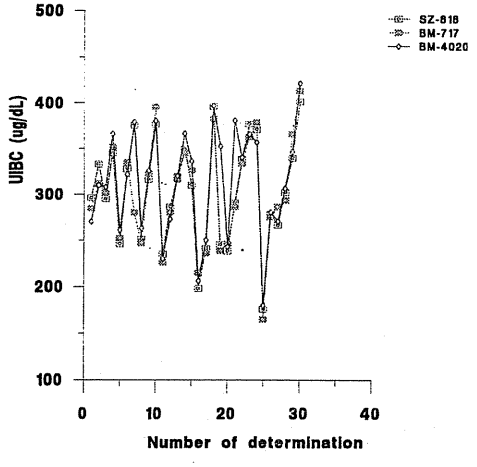
The reagents of TIBC determination were provided from Stanbio (No 0370, USA), including Iron-Ferrozine Color Reagent (CR; ferrozine,14.4 mM), Iron-Binding TRIS Buffer (BB: 0.5 M, pH 8.1), and Iron Standard (IS; ferrous ammonium sulfate, 500 µ g/dl). The control sera of different levels and serum iron determination kit were obtained from Boehringer Mannheim. Thirty samples of different subjects were assayed in addition to commercial sera. The direct, automated application was made to

Table I. *Chemistry Parameters of the Analyzers*

HİTACHİ 717		Z 818.	
Test	UIBC	1. Name	: UIBC
Assay Code	(2Point)-(24)-(50)	2. Method	: 2P-END
Sample Volume	(20) (10)	3. Method	: END-R1
R1 Volume	(200) (100) (NO)	4. WAV 1	: 576 nm
R2 Volume	(50) (20) (NO)	5. WAV 2	: 659 nm
Wave Length	(660) (570)	6. SM VL	: 20 µL
Calib, Method	(LİNEAR) (0) (0)	7 IJ VL	: 100 µL
STD, (1) CONC, POS	(0)-(1)	8. R1 VL	: 200 µL
STD, (2) CONC, POS	(500)-(4)	9. R2 VL	: 50 µL
SD Limit	(0.1)	10. UNIT	: µg/dL
Duplicate Limit	(100)	11. ABS L	: 500.00
Sensitivity Limit	(0)	16. DEC-P	: 0
ABS, Limit (INC/DEC)	(0) INCREASE	17. ABS L	: 500.00
Prozone Limit	(32000) (UPPER)	18. ABS H	: 2.000
Expected Value	(250) (400)	19. N-R L	: 250.00
Panic Value	(0)-(1000)	20. N-R H	: 400.00
Instrument Factor	(1.0)	21. L CHK	: 0
		22. M1STR	: 3m40s
		23. M1STP	: 4m40s
		24. M2STR	: 8m40s
		25. M2STP	: 9m40s
		26. S BLK	: 0.9000
		27. CBLK	: 0N
		28. CRL-A	: 1.0000
		29. CRL-B	: 0.00
		30. M STD	: LINE SEG

two different autoanalyzers: Hitachi 717 (BM 717) and Mitsubishi (SZ 818). The same reagent sets were used in manual determinations in photometer (Hitachi 4020) in order to make the correlation studies. In UIBC determination, the final iron determination was made by ferrozine method with no deproteinization (3).

Fig. 1. *The Correlation Points Of UIBC Determinations In Three Different Analyzers.*



Preparation of the Reagents for the Autoanalyzers: The same reagents prepared were used in the analyzers to which the determination was applied, since they had the same reagent position property and the same measurement principle. In these analyzers, reagent one (R1) was prepared by adding 1 volume of IS to 10 volumes of BB, and CR was used as R2 directly.

Programming: Table 1 shows the chemistry parameters of UIBC determination for two analyzers. The common parameters of two analyzers include method used, sample and reagent volumes, wavelength, standard concentration, and calibration mode.

Calibration: After programmed as in Table 1, the analyzers were calibrated. For this purpose, reagent blank value was entered as zero and no reagent blank measurement was made. To the standard position was placed iron-free distilled water as a standard, since there was iron standard in R1 and this was pipetted into the standard measurement cuvette.

Method Description: Two-point end point method was used for UIBC determination. The ratio of sample volume to R1 volume was 1/10, the same ratio as that of IS/BB in preparation of R1. In this method, the analyzer sampled the calibrator and sera into individual cuvettes and then pipetted the R1. At that point, the saturation of serum with iron in R1 began in the sample (test) cuvettes and nothing

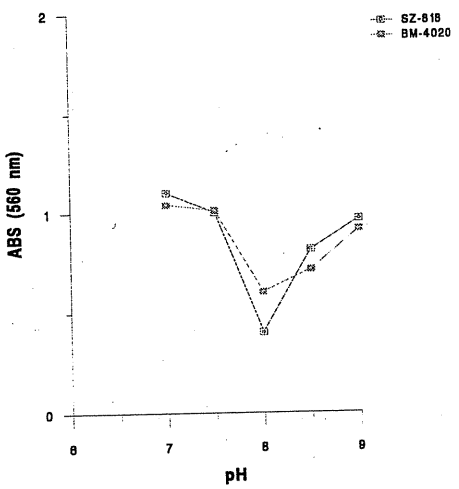
Table II. *Statistical Evaluation of the Results(*)*

	BM 717 (CV %) (X±SD)	SZ 818 (CV %) (X±SD)	BM 4020(CV %) (X±SD)	Other Method (X±SD)	Label Value (µg/dL)
CS-1(n=10)					117(92-142)
Fe	126±7.6	123±7.1	130±7.9	158±5.9	
UIBC	155±7.2(4.6)	148±5.0(3.3)	156±5.1		275(231-319)
TIBC	281±14	271±12	286±13		
CS-2(n=10)					125(99-151)
Fe	128±8.0	136±7.7	127±8.0	128±7.2	
UIBC	124±5.2(4.2)	109±6.2(6.8)	133±9.1(6.8)		262(220-304)
TIBC	252±13	245±14	260±16		
PS(n=30)					
UIBC	305±60	304±58	307±59		

CS : Control serum; PS : Patients' serum
(*) t-test results showed no statistically significant difference between any two analyzers mentioned in the table and between the methods.

occurred in calibration cuvette. At the end of the saturation step, in the test cuvette some free iron atoms were bound by the iron-free transferrin molecules and some left as excess iron in the medium. At this point, R2 (color reagent) was added to all cuvettes, causing a reaction in calibration cuvette by present iron, which included 10 volumes of Tris buffer, 1 volume of iron standard, and 1 volume of distilled water, and a reaction in test cuvette by excess iron, which included 10 volumes of BB, 1 Volume of IS, and 1 volume of serum. The final color was proportional to the excess iron in the test cuvette and was measured at 570 or 576 nm.

Fig 2. *Yhe Absorbance Values Of Reaction Medium At 560 nm In Two Different Analyzers.*



Calculation of the UIBC Results: The analyzer measures the excess iron in the test cuvette by considering the calibrator absorbance. Since we add 500 µg/dl iron together with R1 to the reaction medium of test, 500 minus excess iron (measured by the analyzer) will give UIBC. When UIBC is added to serum iron concentration, the result is TIBC. These calculation must be made by the operator in the case of SZ 818 analyzer and can automatically be made by the BM 717 as follows. In calculated test mode, TIBC =UIBC + Serum Fe; Transferrin Saturation (%)= Serum Fe x 100/TIBC, and the compensated test mode UIBC = 500-UIBC (measured as excess iron) are entered. The SZ 818 prints out the excess iron as UIBC. The operator must subtract the excess iron from 500 and obtain the true UIBC value.

Results and Discussion

The Table 2 shows the statistical evaluation of the results.The correlation analyses were done between BM 717 and SZ 818 analyzers and between the automated and manual assays (Fig. 1) The general procedure for determining UIBC is to saturate the transferrin with excess amount of iron, to remove the unbound iron and to assay the iron. Several methods have been used for determination of UIBC, including adsorption on ion-exchange resin (4-6), resin-treated filter paper (7), magnesium carbonate suspension loaded with ferric iron (8), and adsorption on magnesium carbonate (9). This study represents a direct method, not requiring any pretreatmen of patient's serum for UIBC determination and providing direct entering of UIBC assay as in serum iron determination in discrete sample analyzers. It is well known that the transferrin molecule can bind only ferric, not

Table III. *The Volumes Added To The Test Medium*

	Test cuvette	Standard cuvette
	Automated/manual	Automated/manual
Iron-saturating Tris buffer(BB)(pH: 8.0, 0.5M)	200µL/400µL	200µL/400µL
Iron Standard (IS) (500µg/dL)	20µL/40µL	20µL/40µL
Serum	20µL/40µL	--- ---
Distilled water	--- ---	20µL/40µL
Color reagent (CR) (Ferrozine, 14.4 mM)	50µL/100µL	50µL/100µL

ferrous, ions in physiological pH (10). For determining iron bound to transferrin, it is released from the transferrin in acidic pH and reduced to ferrous state by any reducing agent such as ascorbic acid and hydroxylamine, and reacted with color reagent such as ferrozine (3,8). In previous methods, the ferric ions were used for saturation of transferrin in vitro, and the free ferric ions were reduced to ferrous state and assayed. In automated procedures, it is possible to saturate the transferrin with ferric ions, but after the saturation step, there is a difficulty in reducing the ferric to ferrous state for the formation of colored complex. In order for dissolving this problem, we tried to saturate the transferrin with ferrous ion in different pHs. For this purpose, different solutions of different pHs were prepared: saturating tris buffer 0.5 M of different pHs (7.0, 7.5, 8.0, 8.5, and 9.0). These buffer solutions were used as BB in making R1. The both manual and automated methods showed a maximum saturation of transferrin with ferrous ions at pH 8.0 (Fig. 2), suggesting that the transferrin molecule can bind ferrous ions at basic pH and the ferrous ions may be used for transferrin saturation only at pH 8.0. For the purpose of correlation studies, the same serum samples were assayed in both autoanalyzers and manual analyzer. Just as there was a good correlation between the two autoanalyzers, so a statistically significant correlation was obtained in automated vs manual assays. The correlation coefficients were found as follows: r=0.982 for BM 717 - SZ 818; r=0.980 for BM 717 - BM 4020; and r=0.977 for SZ 818 - BM 4020. On the other hand some of the samples were also assayed with different method, which used magnesium carbonate particles saturated with ferric ions as saturating agent. The results obtained with the latter were compared with our method and it was seen that there was no statistically significant difference between these two methods for UIBC determination(Table 2). Similarly, the serial determinations were made in the same sample in order to obtained the repeatability or intraassay coefficient of variation (CV %). The autoanalytical procedure showed a low CV % (Table 2) or a good reliability of the test. It is imperative that some parameters be entered or same procedures be made in automated assay of UIBC. Table 3 shows the volumes added for automated and manual methods used in this study. In any autoanalyzer which is selective and patient-oriented and has two-reagent addition capability, one sampling and two reagent pipettings are possible. As mentioned before, in this study BB and IS solutions were used in preparing the R1, serum, standard or blank may be placed in sample tray, and the CR can be used as R2. Since in this condition, R1 is added to all cuvettes, we have no chance to add the standard to standard cuvette and we have to place the distilled water in the place of calibrator instead of calibrator. For the same reason, we cannot execute blank study because R1 plus distilled water plus R2 as in the case of blank means a color development higher than test cuvette and equals to standard cuvette defined in this study. On the other hand, the volumes of IS and serum have to be same, otherwise the calibration will be incorrect. We made the manual assay just as in autoanalytic method in order for us to compare with each other. However in any manual procedure one can execute the blank, standard and sample cuvettes. Our overall results show that a simple, reliable determination of serum UIBC can directly be made in an autoanalyzer which is open directly, discrete and selective analyzer.

References

1- Levy A, and Vitacce. Direct determination and binding capacity of serum iron. Clin Chem. 7:241-248, 1961.
2- Goodwin J, el al: Direct measurement of serum iron and binding capacity. Clin Chem 12: 47-57, 1966.
3- Stookey LL. Ferrozine: A new spectrophotometric reagent for iron. Anal. Chem. 42, 779-782, 1970.
4- Peters T, Giovanniello TJ, Apt L. and Ross, JF. A new method for the determination of serum iron-binding capacity. J. Lab. Clin. Med. 48, 274-276, 1956.
5- Cook J D. An evaluation of adsorption methods for measurement of plasma iron-binding capacity. J. Lab. Clin. Med. 76, 497-499, 1970.
6- Lehmann HP and Kaplan A. Use of an anion-exchange column to determine serum iron-binding capacity. Clin. Chem. 17, 491-495, 1971.
7- Giovanniello TJ, DiBenedetto G, Palmer DW, Peters T, Fully and semi-automated methods for the determination of serum iron and total iron-

binding capacity. J. Lab. Clin. Med. 71, 874-877, 1968.
8- Sclavo, Iron TIBC, Cat No 81793, Italy 1992.
9- White MJ and Flashka HA. An automated procedure with use of ferrozine, for assay of serum iron and total iron-binding capacity. Clin. Chem. 19(5): 526-528, 1973.
10-Tietz NW. Blood Gases and electrolytes in Fundamentals of Clin Chem. ed NW Tietz 1976, WB. Saunders Co, Philadelphia, pp 922-923.

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