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Automated stopped-flow analyser in clinical chemistry: determination of albumin with bromcresol green and purple

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Summary

We describe an adaptation of a microcomputer-controlled stopped-flow analyzer for automated analysis of albumin in serum. The immediate reaction between bromcresol green or purple and albumin is used to develop a routine procedure with 12 s reaction time. The approach shows excellent linearity to 68 g/l, good sensitivity, a relative SD of $< 0.5\%$, mean recovery 99.1%, high sample throughput (180 prediluted samples/h) and no significant interferences. The 'reaction time' related overestimation of albumin that appears in prolonged procedures is eliminated, and results with this method correlate well with those obtained using the more specific immunonephelometric method.

Introduction

The stopped-flow technique for the rapid mixing of chemical reagents has gained widespread importance in studies of the rates of rapid chemical reactions. More than a decade ago [1,2] we developed an analytical stopped-flow analyzer (SFA) that has evolved into an increasingly useful instrument for routine or investigative high-speed analytical/clinical chemical determinations. The SFA has the high sample throughput of the air-segmented continuous flow and flow injection analyzers and has some significant advantages. The reagent and sample are precisely measured and quantita-

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tively mixed so that precisions of 0.1% are possible with the SFA. Also, quantitative reaction rate methods that are often more specific than the equilibrium methods can be used readily, even for very fast reactions in the 1-s range. Our most recent microprocessor-based SFA [3] is much simpler, less expensive and more compact than its hardwired or minicomputer-based relatives [4,5]. We use this system for automated fast determination of albumin in serum by the rapid reactions with indicator dyes, bromcresol green (BCG) and purple (BCP).

The BCG method was introduced by Rodkey [6] and improved by Doumas et al [7], but it was realized that results by this method did not compare well with more specific methods when reaction times were prolonged. This positive bias was especially serious in the clinically important lower concentration range. It was shown [8] that the reaction of serum samples with BCG proceeds in two steps. Albumin reacts almost immediately, while some other serum proteins contribute to a much slower reaction.

Recently, several automated procedures based on short reaction times have been proposed [9–16] and their results were in good agreement with those of specific immunological methods. The advantage of the automated SFA for absorbance measurements in precise short times after the mixing of the reagents makes it an atractive system for the determination of albumin. We also describe a procedure using BCP, which has been proposed as a promising more specific reagent for albumin instead of BCG [17,18].

Materials and methods

Apparatus

The automated SFA (Fig. 1) has been developed in our laboratory [3] and it can be used for equilibrium or reaction rate methods with accurate and precise results.

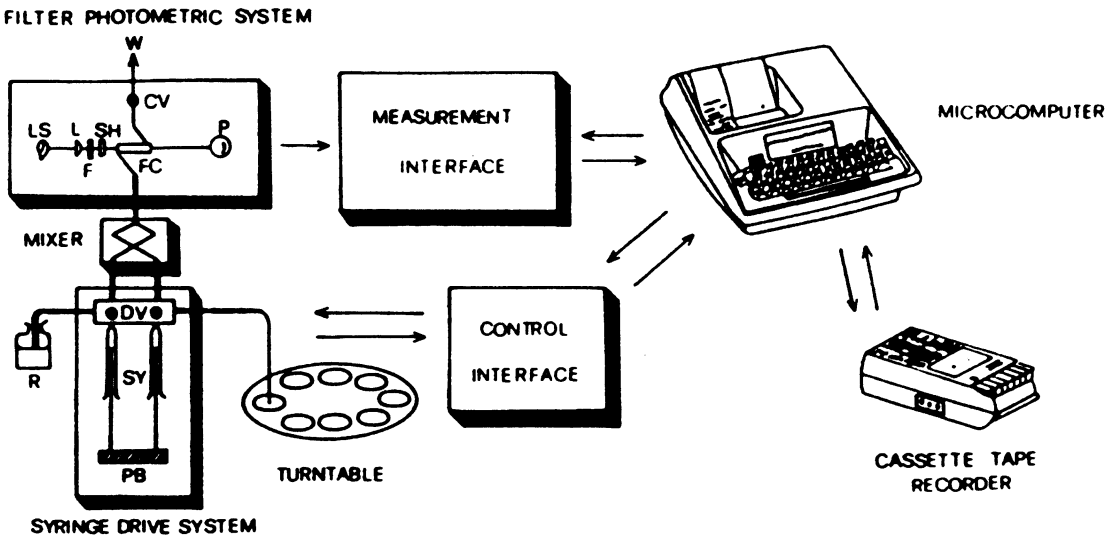

Fig. 1. Automated basic SFA. LS, Light source; L, lens; F, filter; SH, Shutter; FC, flow cell; CV, check valve; W, waste; P, photomultiplier; R, reagent; DV, dual 3-way flow valves; PB, plunger block; SY, syringes.

This system provides for automatic portioning and mixing of prediluted serum samples and reagent and delivery of the mixed solution into the measurement cuvette (1-cm pathlength). About 150 μl of prediluted sample or reagent are delivered by each syringe and four flushes are used to prevent carryover from sample to sample. An AIM 65 (Rockwell International, Anaheim, CA, USA) microcomputer controls all system operations and data aquisition and processing. Several investigative and routine programs have been developed for equilibrium and rate procedures. Interference filters with about 10-nm bandpass at 630 and 600 nm, for BCG and BCP methods, respectively, were used in the photometer. All measurements were carried out in an air-conditioned laboratory maintained at a nominal temperature of 25°C.

Comparative studies were done using a BCG automated procedure in a Technicon SMA II multichannel analyzer. The immunonephelometric assay was carried out with a fluorescence spectrophotometer (Model 512 A, Perkin–Elmer) equipped with a magnetic stirrer.

Reagents

All solutions were prepared in de-ionized water from reagent grade materials, unless otherwise stated.

BCG stock solution, 1.2 mmol/l 168 mg of bromcresol green (Fisher Scientific Co., USA) were dissolved in 10.0 ml of 0.10 mol/l sodium hydroxide and diluted to 200 ml with water. This was stored at 4°C.

Succinate buffer, 0.200 mol/l, pH 4.0 23.8 g of succinic acid were dissolved in about 800 ml of water, the pH was adjusted to 4.0 with sodium hydroxide, and was diluted to 1 liter with water. This was stored at 4°C.

BCG working solution was prepared by diluting 1 vol of BCG stock solution with three volumes of succinate buffer and adding 8.0 ml of 30% Brij-35/l (Sigma, St. Louis, MO, USA). The pH was carefully adjusted to 4.20 ± 0.05. This was stored at 4°C.

BCP-stock solution, 40 mmol/l 216 mg of bromcresol purple (Aldrich Chem. Co., USA) were dissolved in 7 ml of absolute ethanol and when a clear orange solution was obtained it was diluted to 10 ml with ethanol.

BCP working solution was prepared by dissolving 12.6 g of sodium acetate in about 800 ml of deionized water, adding 17.5 ml of 3 mol/l acetic acid solution, 2 ml of 30% Brij-35 and 2.5 ml of BCP stock solution. After diluting to 1 liter the pH was adjusted to 5.20 ± 0.05 using acetic acid or sodium hydroxide solution. The reagent was stable for at least 1 wk at room temperature.

Albumin standards The stock standard was prepared by dissolving 1.00 g of human serum albumin (Fraction V, Sigma containing 96–99% albumin (remainder

mostly globulins and various amount of moisture) in 10.0 ml of physiological saline. The concentration of this solution was established by standardizing against a human albumin reference standard solution of 79.0 g/l, using the under development BCG-SF method, and it was found to be 82.0 g/l. The reference standard was obtained by reconstitution of the content of a vacuum-sealed ampoule of lyophilized pure human albumin (DADE, American Hospital Supply Corp., Miami, FL, USA, Cat. no. B 5158, specified as 100% albumin by moving-boundary electrophoresis and assayed by the macro-Kjeldahl procedure). Serial dilutions with saline diluent were made from this standardized stock to yield nominal concentration of 21.7, 32.6, 43.4, 54.3 and 67.9 g of albumin/l.

Saline diluent Contains 9 g of sodium chloride/l deionized water.

Quality control sera Lyophilized control sera used were 'Validate' Normal and 'Versatol-A' (General Diagnostics, Morris Plains, NJ, USA), 'Moni-Trol' I and II (DADE), 'Chem Trol' Normal and Abnormal (bovine serum, Clinton Labs., Santa Monica, CA, USA). These controls were reconstituted according to the manufacturer's instructions. Serum samples obtained from patients were used in evaluating the method.

Procedures

Automated BCG-SFA

25 μl of standard or serum samples were prediluted with 2.5 ml of saline diluent in 5-ml plastic cups using an automatic pipette. One channel of the SF system was used to portion the BCG working solution and the other one the standards and samples from the turntable. The interactive routine equilibrium program was first loaded from the cassette recorder into the computer's memory. Then, the blank solution was injected into the flow cell, an integration time of 0.5 s was selected and the dark current and 100% transmittance were measured automatically. A delay time of 10 s and a measurement time of 2 s (four integrations) were provided to the program along with the number of standards and samples to be measured, the number of measurements to be averaged, and the number of flushes. The program then sequences through each standard, flushing the system between each standard and prompting the operator for its concentration. After the standards have been measured, the micro-computer calculates the linear least-squares regression line and prints its slope, intercept, correlation coefficient and the SEM of the estimate. Samples are measured, after which the concentration of the albumin in the sample is calculated from the regression line and printed. A dedicated program can also be used with all the information, time parameters and standards concentration, contained in the software. Once the operator inputs the number of samples, the analysis will be completed automatically.

Automated BCP-SFA

The same procedure as the BCG-SFA was used except that 50 μl of standards and samples were prediluted with 4.00 ml of saline.

Correlation studies

We compared the SFA results with those obtained with an automated air-segmented continuous flow BCG procedure [19] with a SMA II computer-controlled multichannel analyzer and with a 5-min reaction time. Comparisons with the more specific immunological nephelometric method performed according to Lizana and Hellsing [20], with minor modifications, were also carried out. Antiserum was obtained from Sigma.

Results

Optimization of the procedures

Optimization of the instrument performance involved choosing the delay and measurement time. The effect of reaction time on the absorbance was studied using an investigation program. The reaction was monitored for 10 min after the mixing of the solutions and 50 points of the reaction curve were printed out. In Table I, absorbance values for albumin standard and abnormal samples are shown for both BCG and BCP reagents. A slow increase of the absorbance occurs with the BCG method that could cause the time-related overestimation of serum albumin if a rather long reaction time is used. With the BCP reagent there is no slow reaction, suggesting that the method is more specific for albumin. This specificity of the new reagent has been proved recently by several researchers by studying the reaction of BCP with albumin-free serum globulin preparation, purified globulins or pure human transferrin solutions and by comparing the results with the widely accepted electroimmunoassay method [18,21–24].

A delay time of 10 s and a measurement time of 2 s were chosen as the optimum time parameters for both BCG and BCP methods as a compromise of keeping the reaction time as short as possible and obtaining good within-run and between-run precision. A shorter delay time gave poor precision because of the generation of a turbidity during the mixing caused by the Brij.

TABLE I

Time-related absorbance values for albumin standard solution and abnomal sera by the BCG and BCP method

Time (min)	Standard (33 g/l)		Sample 1 (19 g/l)		Sample 2 (21 g/l)		Sample 3 (19 g/l)	
	BCG	BCP	BCG	BCP	BCG	BCP	BCG	BCP
0.2	0.237	0.220	0.139	0.145	0.154	0.165	0.139	0.144
1	0.239	0.222	0.145	0.145	0.165	0.165	0.149	0.145
3	0.241	0.222	0.154	0.147	0.178	0.166	0.162	0.147
5	0.242	0.224	0.155	0.148	0.185	0.166	0.168	0.147
7	0.242	0.226	0.161	0.149	0.189	0.166	0.171	0.147
10	0.242	0.228	0.166	0.152	0.192	0.167	0.176	0.147
ΔA	0.005	0.008	0.027	0.007	0.038	0.002	0.039	0.003

TABLE II

Results used for BCG and BCP working curves

Albumin (g/l)	BCG		BCP	
	Absorbance [a]	RSD (%)	Absorbance [a]	RSD (%)
21.7	0.1590	0.3	0.1383	0.5
32.6	0.2351	0.2	0.2091	0.4
43.4	0.3024	0.1	0.2674	0.2
54.3	0.3719	0.2	0.3285	0.2
67.9	0.4565	0.2	0.3975	0.2

[a] Average of 5 measurements. Delay time, 8 s; measurement time, 2 s (4 integrations). Working curves: BCG, slope = 0.006409, intercept = 0.02308, $r = 0.9997$, Sb = 0.000001, Sa = 0.00005 SE of estimate = 0.00004; BCP, slope = 0.00558, intercept = 0.0227, $r = 0.9990$, Sb = 0.00008, Sa = 0.004, SE of estimate = 0.003.

The final BCG reaction conditions (pH, buffer and reagent concentrations) were based on those described by Doumas et al [7] as modified by Gustafsson [8]. For the BCP reaction, final conditions as described by Pinnell and Northam [18] were used. Appropriate modifications of the stock reagents were made to meet the aforementioned final conditions after the 1 : 1 mixing of the working reagents with the prediluted samples in the SFA. These conditions achieved the best compromise between maximum absorbance of the BCG or BCP/albumin complex and minimum reagent absorbance.

Linearity, sensitivity and stability of working curve

Working curves were linear up to 68 g/l for both BCG and BCP methods. The BCG method is more sensitive so that a 1 : 200 dilution in the observation cell is practical. In the BCP method a dilution of 1 : 160 is used. Typical results obtained for the working curves are shown in Table II. The day-to-day stability of the working curve for the BCG method over a period of ten days is shown in Table III.

Precision

Within-run and day-to-day precision of the BCG-SFA method was studied for four commercial control sera (Table IV).

Analytical recovery The accuracy of the proposed BCG-SFA method was checked by adding various known amounts of human albumin standard in twelve normal and abnormal control sera. Analytical recovery ranged from 94.8–104.6% with a mean of 99.1% (Table V).

Interferents No interference was observed from bilirubin at level 1.6 g/l and hemoglobin at level 5 g/l added to a control serum. Also no interference was observed from the following drugs at the given concentrations: indomethacin (24 mg/l); salicylate (2 g/l); diazepam (6.4 mg/l); warfarin (10.4 mg/l); phenylbuta-

TABLE III

Day-to-day stability of BCG working curve

Standard	Absorbance		
	Day 1	Day 5	Day 10
21.7	0.1590	0.1595	0.1608
32.6	0.2351	0.2379	0.2381
43.4	0.3024	0.3069	0.3045
54.3	0.3719	0.3771	0.3741
67.9	0.4565	0.4573	0.4572
Slope	0.006409	0.006430	0.006382
Sb	0.000001	0.000001	0.000001
Intercept	0.02308	0.02482	0.02625
Sa	0.00005	0.00005	0.00004
Corr Coeff	0.9997	0.9993	0.9996
SE est.	0.000038	0.000038	0.000035

zone (0.8 g/l); acetaminophen (64 mg/l); chlorpromazine (16 mg/l); phenytoin (160 mg/l) and gentamicin (64 mg/l).

Comparison with other methods Results by the described BCG-SFA procedure were compared with results by the BCP-SFA, the SMA II BCG (5-min reaction) [19] and the immunological nephelometric method [20]. Fifty to one hundred samples

TABLE IV

Precision of BCG stopped-flow method

Precision	Samples [a]			
	1	2	3	4
Within-run				
No. assays	10	10	10	10
Mean (g/l)	45.1	23.5	44.2	27.4
SD	0.11	0.07	0.13	0.17
CV (%)	0.24	0.29	0.29	0.62
Between-run				
No. assays	10	10	10	10
Mean (g/l)	45.0	23.6	44.3	27.6
SD	0.18	0.10	0.14	0.21
CV (%)	0.40	0.42	0.32	0.76
Day-to-day				
No. assays	10	10	10	10
Mean, (g/l)	44.9	23.6	44.4	27.8
SD	0.27	0.15	0.15	0.39
CV (%)	0.60	0.64	0.34	1.4

[a] Samples: 1, Validate normal; 2, Versatol-A; 3, Monitrol I; 4, Monitrol II.

TABLE V

Recovery data for the determination of albumin in serum by the BCG-SFA method

Sample	Albumin (g/l)			Recovery (%)
	Before standard addition	After standard addition		
		Expected	Determined	
1. Validate normal	43.8	56.8	56.9	100.8
2. Validate normal	43.8	65.5	65.5	100.0
3. Chemtrol normal [a]	41.6	54.6	54.0	95.4
4. Chemtrol normal [a]	41.6	63.3	62.6	96.8
5. Monitrol I	43.8	56.8	57.4	104.6
6. Monitrol I	43.8	65.5	65.5	100.0
7. Monitrol II	27.6	49.3	49.0	98.6
8. Monitrol II	27.6	60.2	59.0	96.3
9. Versatol abnormal	24.0	45.7	46.4	103.2
10. Versatol abnormal	24.0	56.6	57.1	101.5
11. Chemtrol abnormal [a]	27.8	49.5	48.8	96.8
12. Chemtrol abnormal [a]	27.8	60.4	58.7	94.8
				Mean 99.1

[a] Bovine control serum.

selected at random, were analyzed by the compared methods on the same working day. Albumin values were in the range of 21–48 g/l. Statistically summarized data are shown in Table VI, and the correlation curves in Fig. 2. As shown BCG-SFA and BCP-SFA correlated well with an average bias of about 2%. The SMA II (5 min) procedure gave higher results especially in the low albumin concentration range showing systematic positive biases compared to both SFA methods. The two SFA

TABLE VI

Regression equations for the comparison study of stopped-flow methods

y/x	No. specimens (n)	Slope	y intercept	$SD_{regression}$	r
BCG-SFA BCP-SFA	100	0.981	−0.4	1.8	0.944
BCG-SFA SMA(II) (5 min)	100	1.127	−10.0	2.1	0.933
BCP-SFA SMA(II)	100	1.203	−11.0	2.4	0.924
BCG-SFA Immunonephelometric	50	0.967	1.4	1.0	0.973
BCP-SFA Immunonephelometric	50	0.987	1.7	1.1	0.980

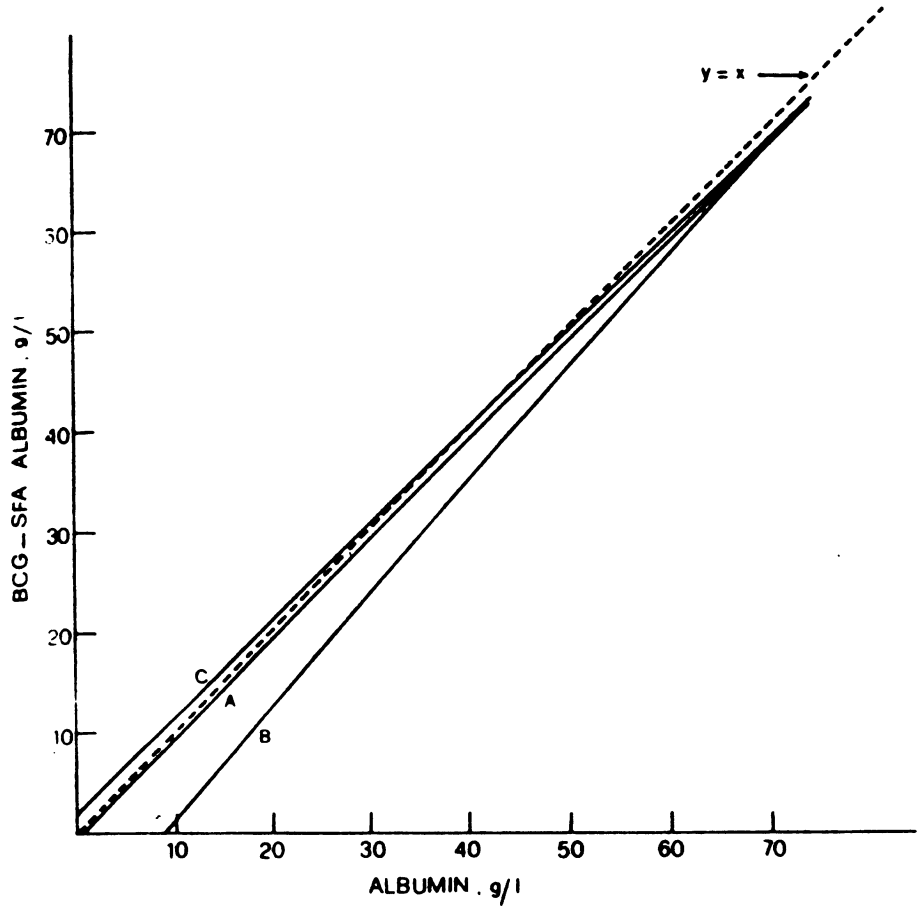

Fig. 2. Results by the BCG-SFA method as compared with the BCP-SFA (A), the SMA(II) 5 min (B) and the immunological nephelometric (C) methods.

methods showed also sufficient agreement with the more specific immunonephelometric method.

Conclusion

The described methods are based on the rapid reaction of BCG and BCP with albumin. Measurement at 10 s is made possible by using the automated stopped-flow system.

The performance of the proposed method is characterized by linearity to 68 g/l, excellent precision (within-run and day-to-day) and 99.1% (mean) of recovery. Only 25 µl of sample are needed for predilution, and the sample throughput is high (180 samples/h), using four flushes and one measurement per sample. The short reaction time eliminates interference from other serum proteins showing slow reaction with the dye-reagents. Comparison studies with the long reaction-time continuous method showed that the reaction time overestimation is eliminated. The BCG-SFA method correlated well with the slightly less sensitive BCP-SFA method, and both showed sufficient agreement with the selective immunonephelometric method. Despite the small discrepancy with the immunological method, the SFA methods are suitable for routine analysis.

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