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SHORT COMMUNICATION / KURZMITTEILUNG

Dye-Binding Method for Rapid Colorimetric Determination of the Cholestasis Characterizing Lipoprotein-X

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Summary: Serum lipoproteins were stained by the addition of Sudan Black B solution. The normal lipoproteins and the excess of Sudan Black were removed with heparin and zinc acetate solution. The abnormal lipoprotein-X remained in the supernatant, the blue colour of which was measured. The empirically obtained constant was used for calculation, thus avoiding standardization.

Farbstoffbindungsmethode zur schnellen kolorimetrischen Bestimmung des für Cholestase charakteristischen Lipoprotein-X

Zusammenfassung: Die Serumlipoproteine wurden durch Zugabe von Sudanschwarz-B-Lösung gefärbt. Die normalen Lipoproteine und der Überschuß an Sudanschwarz wurden mittels Heparin und Zinkacetatlösung ausgefällt. Das abnormale Lipoprotein-X blieb in dem blaugefärbten Überstand, dessen Farbe gemessen wird. Die empirisch ermittelte Konstante würde zur Berechnung benutzt, wodurch die Standardisierung überflüssig ist.

Introduction

The properties and significance of lipoprotein-X were mentioned in the previous paper (1). The value of its quantitative determination, e.g. for assessing the degree of cholestasis and possibly for differentiating between the extra- and intrahepatic types was discussed in *Narayanan*'s review (2). In the present communication the same principle for removing the lipoproteins other than lipoprotein-X was used as previously (1). Sudan Black B was utilized to stain serum lipoproteins of which only the blue-stained lipoprotein-X remained in solution after heparin-Zn^{2+} precipitation. Other lipoproteins, together with the excess of Sudan Black, were removed with the precipitate.

Materials and Methods

Reagents

1. A saturated solution of Sudan Black B (E. Merck, Darmstadt, FRG) in ethylene glycol was prepared by gentle mechanical mixing of 0.5 g of the dye with 100 ml of the solvent for 0.5 h. After about 1 week standing (with occasional mixing) in the dark at room temperature the suspension was centrifuged at about 3000 *g* for 20 min. The supernatant representing a stock solution of Sudan Black was decanted off. To 10 ml of ethanol 50 µl of this Sudan Black solution were added (the tip of the Eppendorf pipette was rinsed out into this volume of ethanol). This ethanolic solution should give an absorbance of 0.35–0.36 at 610 nm (glass cuvette, 1 cm path). If this is not the case, the Sudan Black stock solution should be diluted with ethylene glycol until the indicated absorbance is attained (after the dilution with ethanol). Usually this working solution of Sudan Black in ethylene glycol ist stable for several weeks in the dark at room temperature. Later, when its colour decreases, the more concentrated stock solution should be added to reach the indicated absorbance.

2. Heparin (1 g) and 0.15 mol of NaCl per l of water.

3. Zinc acetate 0.26 mol/l of water.

4. Normal human serum preserved with NaN_3 (30 mmol/l) for the dilution of serum samples with high lipoprotein-X levels; this can be stored for 2 weeks at about 4 °C (not frozen).

Procedure

Each patient's serum was examined in 2 settings in order to avoid the need to repeat the test in the case of high lipoprotein-X levels:

1. 150 µl of the tested serum,
2. 25 µl of the tested serum diluted with 125 µl of normal serum.

To each of the 2 test tubes 200 µl of the working solution of Sudan Black in ethylene glycol were added, mixed well, and after about 5 min (or later) 250 µl of heparin-NaCl were added. The contents were mixed and 50 µl of the zinc acetate solution were added. After thorough mixing the tubes were placed in a water bath at 40 °C for 1 h, then in a bath of 25 °C for 5 min. The tubes were centrifuged for 20 min at about 3000 *g*. To 300 µl of the supernatant 300 µl of water were added and the blue colour (in the case of positive sera) was measured at 610 nm in a Sarstedt plastic cuvette (Nümbrecht-Rommelsdorf, FRG), light path 1 cm, width 0.4 cm against water blank (Spekol 10, C. Zeiss, Jena, GDR). The colour was stable for several hours. Only when the absorbance of the undiluted sample exceeded 1.00 was the absorbance of the diluted sample taken in account (after its multiplication by 6).

The absorbance was converted to g/l lipoprotein-X by multiplication by the constant 1.92 (± 0.047, S.D.). This constant was obtained from 9 calibration curves (with a total of 54 standards) using the concentrated lipoprotein-X standard solution (3) diluted serially with normal serum (fig. 1).

The calibration curve was linear up to the lipoprotein-X concentration of 2.0 g/l. When higher concentrations occur in serum the sample must be diluted with normal serum (and the

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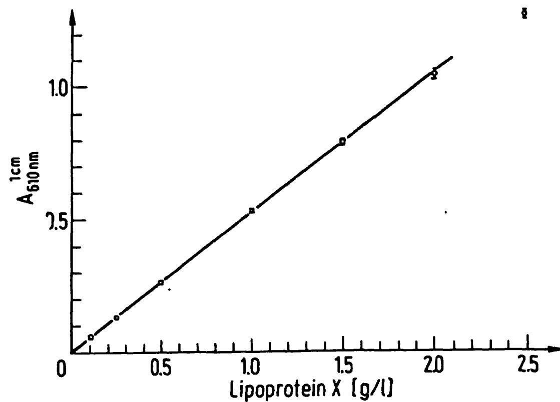

Fig. 1. Calibration curve.

Absorbance values at 610 nm. The lipoprotein-X solution of a known concentration (3) was added in increasing amounts to normal pooled serum to give lipoprotein-X levels indicated on the abscissa. Each absorbance value (empty circles) is the arithmetic mean of 9 analyses. The vertical bars indicate ± S.D. (The absorbance values pertaining to the 2.5 g/l level were not included in the calculation from which the constant 1.92 was derived.)

analysis repeated). The given constant holds only for the Sudan Black working solution of the colour density range indicated above. Lower Sudan Black concentrations gave lower absorbances for the same lipoprotein-X level. Thus, e.g., at an absorbance of 0.31 for the diluted Sudan Black, 1 g/l lipoprotein-X gave an absorbance of only 0.49, whereas 0.52 was obtained with the correct concentration of the Sudan Black working solution (absorbance of 0.35–0.36 when diluted with ethanol).

Results and Discussion

Sera from normal persons and sera from various disease states were investigated by the described method and the results were compared with the enzymatic method (1) and with agar gel electrophoresis (4) (tab. 1).

Both the enzymatic (1) and the present method correlated well (for 28 positive sera: $r = 0.9997$, enzymatic mean $\bar{x} = 4.14$ g/l, present method mean $\bar{y} = 4.01$ g/l, $y = 0.971\,x - 0.03$).

One of the serum samples was analyzed 10 times in one run giving 1.25 ± 0.01 g/l (CV = 0.80%) and (preserved with NaN_3 at 4 °C) on 10 consecutive days with the result of 1.24 ± 0.016 g/l (CV = 1.29%).

To 4 sera (0.16, 0.33, 0.58, 0.99 g/l) lipoprotein-X was added (1.50, 1.0, 0.98, 0.50 g/l) which caused an increase of the levels (to: 1.54, 1.38, 1.67, 1.52 g/l, resp.) thus giving recoveries of 101 ± 6.8% (S.D.).

Tab. 1. Replicate (n) lipoprotein-X analyses by the Sudan Black colorimetric method, by the enzymatic method (1) and by agar electrophoresis.

Diagnosis	Patient	Lipoprotein-X (g/l) Sudan Black procedure n	Mean ± S.D.	CV (%)	Enzymatic procedure	Agar electrophoresis
Control healthy	–	1	not detectable		n.d.	0
Plasmocytoma	H.V.♂	4	not detectable		n.d.	0
Carcinoma coli	T.O.♀	1	not detectable		n.d.	0
Cholecystolithiasis	V.A.♂	2	not detectable		n.d.	0
Hepatitis chronica	O.B.♀	4	not detectable		n.d.	0
Cholecystolithiasis	H.E.♂	1	not detectable		n.d.	0
Decompensatio cordis	B.F.♂	4	0.05 ± 0.002	4.00	0.06	0
Cholecystolithiasis	P.O.♀	5	0.04 ± 0.002	5.00	0.07	0
Status post cholecystectomy (1 d)	N.H.♀	4	0.03 ± 0.001	4.70	0.04	0
Schizophren., cholestasis intrahep. p. chloropromazine	D.K.♂	4	0.24 ± 0.008	3.33	0.26	±
Cholecystitis, choledocholith. susp.	A.A.♂	4	1.16 ± 0.011	0.95	1.26	+
Colica biliaris susp.	Š.V.♂	4	0.08 ± 0.003	3.75	0.09	±
Colica biliaris	N.M.♀	4	0.26 ± 0.009	3.46	0.27	±
Colica biliaris recidiv.	P.M.♀	4	0.51 ± 0.010	1.96	0.51	±
Cholecystolithiasis	P.R.♂	3	0.15 ± 0.006	4.00	0.24	±
Choledocholithiasis	H.A.♂	5	0.21 ± 0.009	4.30	0.31	±
Choledocholithiasis	E.D.♀	4	4.86 ± 0.078	1.60	4.97	++
Choledocholithiasis	K.O.♀	4	14.85 ± 0.101	0.68	15.15	++++
Choledocholithiasis	S.R.♀	4	2.62 ± 0.018	0.69	2.86	+
Choledocholithiasis	M.I.♂	4	2.91 ± 0.014	0.48	3.00	++
Choledocholithiasis	F.A.♀	4	1.13 ± 0.005	0.44	1.27	+
Choledocholithiasis	Ne.M.♀	4	1.12 ± 0.020	1.79	1.23	+
Choledocholithiasis	F.E.♀	4	6.68 ± 0.102	1.53	6.88	+++
Choledocholithiasis (2 d after surgery)	K.R.♂	4	3.42 ± 0.034	1.00	3.34	++
Choledocholithiasis (2 d after surgery)	S.P.♂	4	3.24 ± 0.058	1.79	3.41	++
Choledocholithiasis (1 week after surgery)	K.J.♂	4	0.12 ± 0.003	2.50	0.13	±
Choledocholithiasis (1 d after surgery)	J.R.♀	4	0.46 ± 0.010	2.17	0.48	±
Atresia viarum biliarum	K.L.♂	4	10.56 ± 0.000	0	10.76	++++
Carcinoma pancreatis	M.K.♀	4	5.94 ± 0.039	0.66	5.52	++
Tumor reg. perihepat.	Š.K.♀	4	24.14 ± 0.171	0.71	24.86	++++
Tumor capitis pancreat.	M.A.♂	8	1.62 ± 0.028	1.73	1.84	+
Tumor reg. infrahep.	H.A.♀	4	18.78 ± 0.160	0.85	19.75	++++
Tumor viarum biliarum	Ho.M.♀	4	0.41 ± 0.004	0.98	0.48	±
Tumor capitis pancreat.	P.J.♂	4	6.76 ± 0.102	1.51	6.81	+++

Bilirubin and drugs did not interfere. The limitations, i.e. excessive hyperlipaemia, were discussed in the previous communication (1).

This method is the most simple and rapid one of the hitherto described procedures for lipoprotein-X determination. Possibly, it is easier to perform than the conventional qualitative electrophoretic test. It makes it possible to estimate lipoprotein-X even at very low serum levels with reasonable accuracy and with the use of the simplest materials.

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