INTERFERENCE IN AUTOANALYZER ANALYSIS

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ABSTRACT

This paper presents certain simple procedures for assessing the most common types of interference, due to haemolysis, icterus or lipaemic serum in 19 routine Clinical Chemistry tests and suggests steps to overcome the problem in some tests. A change in the measured concentration, to be analytically significant, had to exceed 2.8 X % coefficient of variation (cv) of the intra-assay analytical variation of each assay. Haemolysis caused interference in 10 of the 19 assays investigated. A haemolysate haemoglobin concentration of 0.29 g/dl, visible to the eye, caused an analytically significant increase in creatinine kinase MB subunit (CKMB), lactate dehydrogenase (LDH), total protein, triglyceride, uric acid and urea, and a significant decrease in alkaline phosphatase (ALP), and total bilirubin. A higher concentration of haemoglobin (0.68 g/dl) caused an additional significant increase in CK, and a decrease in direct bilirubin. Addition of bilirubin caused interference in all the peroxidase linked reactions as well as in the creatinine assay. At a serum concentration of 5.2 mg/dl it caused a decrease in creatinine, glucose, triglyceride and uric acid. At a higher concentration (15.9 mg/dl) it also decreased cholesterol. Lipaemia interference affected the least number of assays. An added triglyceride of 537- 561mg/dl caused an increase in glucose, uric acid, and amylase. At a level of 1122 mg/dl it also increased CKMB, and at a value of 2244 mg/dl it increased total and direct bilirubin. At the highest levels of haemolysis and lipaemia, the serum glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) gave erratic results. Overall uric acid and CKMB were the analytes most susceptible to interference, while serum calcium and phosphate did not suffer from any. The interference depends on the exact assay conditions used and the susceptibility of each individual laboratory's tests should be determined by them. The reasons for the interferences described are discussed.

KEY WORDS: Bilirubin, Haemolysis, Icterus, Assay interference

INTRODUCTION

The most common types of interference in routine clinical chemistry tests are caused by haemolysis, icterus and lipaemia. In the era of manual methods and before discrete analysers were widely used, the measurement of severely haemolysed, icteric or lipaemic specimens was possible using blank corrections, deproteinization and dialysis on continuous flow analysers. The limitations of discrete analyzers

in their susceptibility to interference are well recognized.

Interference in routine clinical chemistry analysis has been defined in different ways. Chemical interference can be defined as "the effect of a substance present in the sample that alters the correct value of the result usually expressed as concentration or activity for an analyte" (1). According to Castano-Vidriales (2), the definition of chemical encompasses drugs, metabolites, contaminants, intracellular constituents, the solution matrix etc., and "chemical

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interference can be defined as a deviation from the result expected for a given concentration of analyte caused by the presence of another chemical species in the sample." The International Union of Pure and Applied Chemistry (IUPAC) (quoted in ref. 2) have defined it as follows "an interfering substance in an analytical procedure causes, at a given concentration, a systematic error in the analytical result. In the case of a quantitative determination an error attributable to interference has to be greater than a multiple of the SD obtained in the absence of the substance (a value of 3 SD is used).

The classical approach to evaluate a new method is to add the interferent material directly to a large pool of serum and measure the results. This is limited by the fact that the matrix of the pooled sample may not represent a typical pathological specimen and the substance added may not be identical to the interferent in the pathological specimen. Alternatively a series of icteric, turbid and haemolysed specimens may be analysed by the method to be studied and by an established method, free from such interferences. This can best be done by analyzing a series of samples that vary only in the concentration of the interferent and studying the linear and non-linear regression line. This model assumes that all interference is independent of the concentration of the analyte, which is not the case. Interference may be analyte-dependent, analyte-independent or a combination of both (1). It may also be defined as similar (chemical or spectral), different (analyte dependent), or non-specific (2), with matrix effects being a composite of all the interferences caused by the presence of different anions, cations and organic constituents of the sample.

As instruments and analytical methods improve in India, individual instruments and methods should be evaluated for their susceptibility to these and other types of interference. There has been a tendency to assume that good analysers produce good results, but actually the results are only as good as the analytical methods in use. Analysers cannot correct for deficiencies inherent in methodology. For example the serum uric acid assay uses an enzymic method

where uricase has great specificity for uric acid, it is however ruined by having a non-specific detector reaction with peroxidase (3). This paper presents a series of simple procedures for assessing interference in routine clinical chemistry tests and suggests steps to overcome interference in some tests.

MATERIALS AND METHODS

The BM/Hitachi 912 analyser (Boehringer Mannheim, Germany) was used to carry out all the measurements, except for glucose and amylase which were performed on the Hitachi 911 analyser. All the 19 routine chemistries were carried out using procedures and reagents described by BM in their methodological literature (see Appendix for details and full list of abbreviations used for each test). The alternative method used to measure creatinine in specimens with a high bilirubin was the Boehringer Mannheim HiCo creatinine twin mode assay, using two channels on the autoanalyser. Both are kinetic reactions and the blank rate is used to compensate the test rate. The methods described below are standard procedures used to prepare haemolysate etc, and were derived from those of Kazmierczak et al (4). Pooled serum for the studies were prepared by mixing clear sera, previously analysed and known to have normal or near normal values for the analytes to be tested. This material must be assumed to be potentially infectious and treated with appropriate caution. In later studies clear previously analysed sera with increased values of some analytes to be assessed were used. Initially two concentrations of all the interferents were prepared, later pools with 3 levels of triglyceride and 4 levels of increasing bilirubin were prepared. All the analyses were performed on the same day as the interferent pools were prepared.

Preparation of sera with increasing haemolysis

The influence of haemolysis was studied by adding haemolysate to a pool of human sera. Five ml of lithium heparinate blood was centrifuged for 10 mins at approximately 3000rpm., and the plasma separated. A volume of 0.4 ml of the red cells was taken and 4 ml of 0.9% saline added. The mixture

was shaken and again centrifuged for 10 minutes. The washing process was repeated twice. The supernatant was removed, then 2ml of distilled water added to the cells. After leaving for 15 mins at 4°C to disrupt the red cells, the mixture was centrifuged for 10 minutes to remove cell debris. A small sample of the haemolysis diluent was analysed for haemoglobin, then the haemolysate added to the serum pool according to the following scheme.

Tube number	1	2	3
Haemolysate (μ l)	-	30	70
0.9% NaCl (μ l)	100	70	30
serum pool (μ l)	900	900	900

Preparation of sera with increasing concentrations of bilirubin

To study the influence of hyperbilirubinaemia a serum or plasma pool has to be spiked with a bilirubin solution. Note the serum pool used will have bilirubin present already, this will be the base value. Approximately 15 mg of bilirubin was weighed in a vial protected from light, 1.25 ml of 0.1 M NaOH was added and it was left for 5 mins to dissolve. After dissolution of the bilirubin, 3.75 ml of distilled water was added and the solution protected from light using aluminium foil. It was used as soon as possible. The bilirubin solution was added to a serum pool according to the following scheme :

Tube number	1	2	3	4	5
Bilirubin (μ l)	-	20	50	70	100
0.9% NaCl (μ l)	100	80	50	30	-
serum pool (μ l)	900	900	900	900	900

In the case of bilirubin and haemoglobin the interferent was in a fixed volume 100 μ l to 900 μ l of base serum, so tube 1 is directly comparable with the other tubes. In the initial study only tubes 1,2 and 5 were used.

Preparation of sera with increasing concentrations of triglyceride

A 1:10 dilution of a 10% fat emulsion (intralipid) obtained from Fresenius Mafatal Medical Ltd, Pune was prepared using pooled serum. This gives the

Intralipid pool, the triglyceride content of which is reported to be 62.5mM ie 5500 mg/dl. The stock solution was then diluted 1:2, 1:4, and 1:8 with the same serum pool. A serum base was prepared by diluting the serum pool 9:10 with saline.

Tube number	1	2	3	4
Intralipid pool (μ l)	-	50	100	200
serum pool (μ l)	450	350	300	200
saline (μ l)	50	-	-	-

For the initial study the intralipid pool was prepared as a 7:100 dilution (70 μ l Intralipid was made up to 1ml with a serum pool). The dilutions of this lipid solution were 3:7 and 1:7 with the serum pool. Since the upper detection limit of the TG assay is 1000 mg/dl, the apparent triglyceride concentration of the tubes was obtained from the 1:7 or 1: 8 dilution of the intralipid pool and the concentration of the other pools is worked out as multiples of that value. The ratio of serum pool to intralipid pool could not be kept constant so tube 1 does not exactly resemble the pools with added lipid.

RESULTS

In the initial interference studies the haemolysate pool haemoglobin concentration was 9.7 g/dl, and the bilirubin spiking solution approximately 250 mg/dl. In the initial studies, using two concentrations of interferent, all the analyte levels in the base sera were within normal limits except for creatinine 2.6 mg/dl and urea 44 mg/dl. The values for analytically significant changes in analytes as a percentage of method CV are shown in the Appendix. The data for analytes which were significantly affected by haemoglobin are plotted as an interferograph (5) in figure 1. The final/original value of each analyte result, expressed as a percentage is plotted against the increasing concentration of interferent. This allows direct comparison between the different analytes.

The analytes where there was a significant interference from bilirubin in the first study were CREA, CHOL, GLU, TG and UA. TP showed a significant decrease to 92.4% of its original value and CKMB increased to 140% of its original value at a bilirubin concentration of 23.5 mg/dl. These changes were not

Fig. 1. Interferogram of the effect of increasing haemolysis on analyte results

Haemolysate concentrations were 0.29 and 0.68 g/dl

—◇— 1 = UREA, —▲— 2 = ALP, —×— 3 = BIL-T, —×— 4 = D-BIL,
—○— 5 = CK, —+— 6 = TG, —□— 7 = TP, —▢— 8 = UA,
— — 9 = LDH, 10 = CKMB

Note: the effect of added haemolysate on LDH (100, 237, 367%), and on CKMB (100, 670, 1290%) is not shown completely

further investigated in the second study. For triglyceride interference in the initial study the analytes where a significant change was seen were AMY, BIL-T, D-BIL, CK, CKMB, GLU, LDH and UA. The second study was a more detailed study of bilirubin and triglyceride interference on these respective analytes using a series of 4 increasing bilirubin concentrations and 3 increasing triglyceride concentrations. In the second bilirubin study, the concentration of uric acid (9.4 mg/dl) and glucose (214 mg/dl) was abnormal. The activity of amylase in this triglyceride study was also abnormal (259 U/l).

In the first study, GOT and GPT activity measurement at the highest level of haemolysis was not reliable because the absorbance exceeded the analyzer limit of 3.3. The initial values in the base

serum pool of both of them were <30 U/l. These values fell by 5 U/L at the highest level of bilirubin interference. The subsequent bilirubin study used a serum pool with much higher levels of GOT and GPT (363 and 288 U/l). Here no change was observed in activity, even at a bilirubin concentration of 24.2 mg/dl. With the highest value of triglyceride (2148 mg/dl), the GOT and GPT could not be quantitated accurately, because of erratic readings. LDH activity was significantly decreased to 91% of its original value with a triglyceride concentration of 2148 g/dl in the initial study. However in the second study values rose to 113% of its original value with a triglyceride concentration of 561 mg/dl and upwards. Since these changes were not consistent they were not included. The data from second study of interference from bilirubin and triglyceride, where there was a significant change in analyte concentration are plotted in figure 2-3 as interferographs (5). Table 1 is a summary of the data from all the studies, showing the concentration of interferent above which a significant effect was observed on the analytes listed.

Fig. 2. Interferogram of the effect of increasing bilirubin on analyte results

Bilirubin concentrations were 0.3, 5.2, 10.9, 15.9 and 23.3 mg/dl

—◇— 1 = CREA —□— 2 = CHOL —▲— 3 = TG
—×— 4 = UA —×— 5 = GLU

Fig. 3. Interferogram of the effect of added triglyceride on analyte results

Triglyceride concentrations were 106, 561, 1122 and 2244 mg/dl

Note : the effect of added triglyceride on uric acid (100, 213, 333 and 538%) is not shown completely

—◇— 1 = AMY, —□— 2 = BIL-T, —△— 3 = D-BIL,
—×— 4 = CKMB, —*— 5 = GLU, — 6 = UA

Further experiments were carried out on the measurement of creatinine by the existing method and a new method not susceptible to bilirubin interference. Twenty one specimens with a bilirubin <3.0 mg/dl were analysed, and 18 specimens where the bilirubin was > 14.2 mg/dl. This data is plotted in

Fig 4a. Relationship between creatinine methods in specimens with a total bilirubin <3mg/dl

figure 4(a) and 4(b). There was a good agreement between the two methods when the bilirubin was low, but the existing method has a negative bias at a high bilirubin concentration.

DISCUSSION

The simplest way to present all the data presented in this study is by using interferograms (5). They can be used to compare different assays on the same analyzer or the same assay on different analysers and have generally been derived by evaluating the effect of the interferent on only one concentration of analyte.

Table 1. Summary of point at which analyte changes become significant with increasing haemolysis, bilirubin and triglycerides.

Haemolysis		Bilirubin		Lipaemia	
0.29 g/dl	0.68 g/dl	5.2 mg/dl	15.9 mg/dl	537 mg/dl	1122mg/dl
ALP ↓	CK ↑	CREA ↓	CHOL ↓	GLU ↑	CKMB ↑
BIL-T ↓	D-BIL ↓	GLU ↓	23.5 mg/dl	UA ↑	2244 mg/dl
CKMB ↑		TG ↓	TP ↓	561 mg/dl	BIL-T ↑
LDH ↑		UA ↓		AMY ↑	D-BIL ↓
TP ↑					
TG ↑					
UA ↑					
UREA ↑

Fig 4b. Relationship between creatinine methods in specimens with a total bilirubin >14mg/dl

In this study usually only analyte concentrations within the normal range were used, except for creatinine, glucose, GOT, GPT and uric acid where different concentrations were evaluated. Extrapolation of the effect of an interferent from one concentration of analyte to other concentrations may be highly inaccurate and misleading when applied to analytes for which interference is dependent on the analyte concentration(4).

In this study, the addition of increasing concentrations of bilirubin (5.2 mg/dl) to pooled sera has a significant negative effect on the concentration of creatinine, uric acid, triglyceride, and glucose. There is an effect on cholesterol from a bilirubin of 15.9 mg/dl upwards. Except for creatinine, these are all analysed by peroxidase-coupled enzymatic methods. Both conjugated and unconjugated bilirubin have a strong absorbance between 400-500nm, and can interfere in analyses carried out between these wavelengths. In addition to a positive spectral interference there is a greater negative interference, due to the consumption of hydrogen peroxide or a chromogen intermediate, by the bilirubin. The uric acid assay used here was the most subject to interference, and its measurement has been reviewed in detail (3). It is stated that the presence of potassium ferrocyanide may overcome interference by causing hydrogen peroxide to oxidize ferrocyanide to ferricyanide. This ferricyanide then oxidizes the chromogen via a phenol intermediate that does not react with bilirubin (3). In

the case of glucose analysis, a study was carried out (6) which suggests the incorporation of potassium ferricyanide and bilirubin oxidase in the assay mixture. In practice we need to be aware of the existence of bilirubin interference and take great care in reporting affected results to the requesting clinician.

Interference of bilirubin in the determination of creatinine by the Jaffe reaction is also well documented (7). Bilirubin is oxidized to biliverdin by the alkaline conditions and causes a decrease in absorbance at 510nm. The magnitude of the interference is dependant on the bilirubin concentration regardless of the creatinine concentration. So the greatest interference is at low creatinine concentrations. The magnitude of decrease depends on the bilirubin concentration, not the creatinine concentration i.e interferent dependant. It was analytically significant (-0.3 mg/dl) for a measured creatinine of 2.6 mg/dl, but even though the change was bigger (-0.6 mg/dl) in a sample with a base creatinine of 13.5 mg/dl, this was not analytically significant as a percentage. Autoanalyser methods using a kinetic creatinine assay are prone to this interference. It is necessary to be able to take a kinetic serum blank during the course of the reaction eg Boehringer Mannheim HiCo creatinine assay. HPLC and Kodak Ektachem methods are also unaffected by bilirubin. If the assay is carried out manually on these specimens, addition of acid at the end of the reaction destroys colour produced by creatinine and overcomes this problem.

Kroll et al (1) assessed haemoglobin interference in 3 different assays. It was either independent of analyte concentration (creatinine), dependent on analyte concentration (total bilirubin) or a combination of both (direct bilirubin). In this study, there was no effect of haemolysis on the phosphate concentration of the pooled sera even at an Hb concentration of 0.68 g/dl., even though phosphate is released from haemolysis of red cells. The effect on ALP assays is probably also due to spectral interference at the assay wavelength of 415nm. When haemoglobin is oxidized to methHb it causes a decrease in absorbance at 340nm, so it can effect any method using this wavelength. This explains the absorbance >3.3 in the

GOT and GPT assays at a haemoglobin concentration of 0.68 g/dl, which made the results unreliable.

The mechanism of haemoglobin interference in the bilirubin assay is described by Westwood (8). It may interfere chemically causing destruction of bilirubin prior to reaction, directly interfere in the diazo coupling reaction, and destroy the formed azopigment. All these effects vary depending on the exact assay conditions. The increase observed in the TP assay corresponds almost directly to the increase in haemoglobin protein concentration, but the explanation for the effect of haemolysis on albumin and urea is not clear, it is either spectral or chemical or a combination. Brady and O'Leary (10), have documented interference from haemoglobin in a wide range of assays on a number of automated instruments by distributing a quality control specimen containing 0.5 g/dl haemoglobin.

Lipaemia is visible in serum because of the light scattering effects of VLDL particles and chylomicrons. The turbidity causes a high background reading in many assays, especially those using only a single reading at the end of the assay (1 point). This will cause a falsely high reading because no blank correction is available. However this can be overcome by measuring a true sample blank in the course of the reaction or use of a 2 reagent procedure that takes an early reading of the specimen mixed with the first reagent 1, before the addition of the second reagent and the reaction proceeds. The assay most prone to this interference is uric acid. The problem can be overcome using commercial assays with a lipid clearing factor in the reagents. Filtration of lipaemic specimens through a 0.45µm cellulose nitrate filter,

followed by filtration through a 0.1µm filter may remove the background interference of chylomicrons and commercial lipid clearing solutions are also available for this purpose.

Lipaemia also causes displacement of plasma water, giving falsely low values for sodium in flame photometry methods. Many enzymic methods incorporate detergents which clear lipoproteins and will progressively alter the absorbance rate being measured. One of the assays affected by this clearing is triglyceride itself, since lipase will progressively clear lipaemic specimens causing a decrease in absorbance. As the wavelength being monitored decreases, the apparent absorbance due to lipaemia increases. According to Brady and O'Leary (11) single bichromatic autoanalyser methods are very poor in measuring lipaemic samples and a kinetic procedure or method incorporating a true sample blank is better. They studied the effect of lipaemia 1000mg/dl on a wide range of assays and automated instruments.

The effect of paraproteins, drugs and the whole area of interference in immunoassays will not be discussed here, although they may have a major interference in assays. Interference in clinical chemical analysis has been reviewed by Saibaba et al (12). The clinical biochemist must always be on the alert for possible assay interferences, which might compromise the quality of service being provided. When using commercial methods, kit inserts usually alert the user to possible interference. Diluting the specimens with saline may reduce the concentration of interferent to below a level at which it can chemically affect the assay, but will not reduce spectral or background interference as the ratio of concentration

Appendix. Analytical Significance of each assay expressed as 2.8 X intra assay

Analyte method	coefficient of variation		Units
	%cv	%cvX2.8	
Albumin (ALB)	1.5	4.2	g/dl
Alkaline phosphatase (ALP)	2.5	7.0	U/L
Amylase (AMY)	3.5	9.8	U/L
Total bilirubin (BIL-T)	9.0	25.2	mg/dl
Direct Bilirubin (D-BIL)	9.0	25.2	mg/dl

Appendix continued

Analyte method	coefficient of variation		Units
	%cv	%cvX2.8	
Calcium (CA)	2.5	7.0	mg/dl
Cholesterol (CHOL)	4.0	11.2	mg/dl
Creatine kinase (CK)	5.0	14.0	U/L
CKMB	5.0	14.0	U/L
Creatinine (CREA)	3.0	8.4	mg/dl
Glucose (GLU)	2.0	5.6	mg/dl
Glutamate xaloacetate Transferase (GOT)	2.0	5.6	U/L
Glutamate pyruvate Transferase (GPT)	2.0	5.6	U/L
Lactate dehydrogenase (LDH)	1.6	4.5	U/L
Phosphate (PHOS)	2.5	7.0	mg/dl
Triglyceride (TG)	4.0	11.2	mg/dl
Total Protein (TP)	1.0	2.5	g/dl
Uric Acid (UA)	4.5	12.6	mg/dl
Urea	2.0	5.0	mg/dl

AMY, BIL-T, D-BIL, CK, CKMB and LDH data was worked out from the inter-assay variation.

Method Principles

ALB	:	colorimetric endpoint bromocresol green
ALP	:	colorimetric, kinetic, p-nitrophenylphosphate with AMP buffer
AMY	:	colorimetric, kinetic, ethylidene protected maltoheptaoside substrate (EPS) with 100% chromophore liberation by α -glucosidase.
BIL-T and D-BIL	:	colorimetric, endpoint, Jendrassik-Grof, diazotized sulphanilic acid $\pm$ caffeine activator.
CA	:	colorimetric endpoint, o-cresolphthaleine complexone
CHOL	:	colorimetric, total, enzymatic, endpoint with esterase, oxidase and peroxidase
CK	:	UV, kinetic, N-acetyl cysteine activated
CKMB	:	UV, kinetic, N-acetyl cysteine activated, antibody inhibition of CK-M moiety
CREA	:	colorimetric, kinetic, Jaffe, alkaline picrate
GLU	:	colorimetric, enzymatic (Trinder), glucose oxidase, peroxidase
GOT	:	UV, kinetic, LDH, MDH, aspartate
GPT	:	UV, kinetic, LDH, alanine
LDH	:	UV, kinetic, pyruvate
PHOS	:	UV, endpoint, ammonium molybdate
TG	:	colorimetric, enzymatic, endpoint, lipase, glycerokinase, glycerol phosphate oxidase, peroxidase
TP	:	colorimetric, endpoint, alkaline biuret reagent
UA	:	colorimetric, enzymatic, endpoint, uricase, peroxidase
UREA	:	colorimetric, enzymatic, endpoint, Berthelot, urease, hypochlorite

of interferent: analyte remains unaltered. very important in overcoming problems.
Understanding the mechanism of the interference is

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