CASE STUDY

False positive paracetamol results due to interference in a colorimetric assay. A case study

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ABSTRACT

A 31-year-old female with no medical history of liver disease or intake of herbal medicine was admitted to hospital for abdominal pain, decreased appetite, malaise, tea coloured urine and confusion. Routine laboratory testing revealed elevated liver function tests (LFTs) and marked coagulopathy consistent with a diagnosis of acute liver failure (ALF). Plasma paracetamol level at 121 µmol/L prompted clinical consideration of paracetamol drug overdose; however, the patient denied taking paracetamol. Over the course of her hospitalisation general conditions and liver functions improved; however, plasma paracetamol concentration remained greater than 100 µmol/L . This article discusses the case where the colorimetric principle for paracetamol quantification gave rise to a falsely elevated paracetamol, confounding the clinical picture to suggest paracetamol overdose and recommendations for laboratories using the colorimetric principle to help identify this interference.

Key words: acute liver failure, paracetamol, acetaminophen, coagulopathy, hepatic necrosis, colorimetric.

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CASE

A 31-year-old female, with no medical history of liver disease or intake of herbal medicine, was admitted to hospital for abdominal pain, decreased appetite, malaise, and confusion. Routine laboratory testing of blood sample shown in Table 1 revealed elevated LFTs and marked coagulopathy consistent with a diagnosis of ALF. Plasma paracetamol levels at 121 µmol/L (therapeutic range up to 100 µmol/L) prompted clinical consideration of paracetamol drug overdose, but the patient denied taking paracetamol. Over the course of her hospitalisation general conditions and liver functions improved, however plasma paracetamol concentration remained greater than 100 µmol/L. Failure of the liver to metabolise the drug was suspected and liver transplantation was considered for treatment (1).

Acute liver failure

ALF is defined as a rapid loss of liver function, which in turn results in impaired protein synthesis, and is evidenced by coagulopathy. It can ultimately result in necrosis of hepatic cells and death of the patient if left untreated. Common causes of ALF, but not limited to this list, include:

- Toxic injury (e.g., paracetamol, natural products, isoniazids)
- Idiosyncratic reaction to medical treatment; e.g., antibiotics, NSADs, statins (2)
- Alcoholic and autoimmune hepatitis
- Viral hepatitis: Hepatitis B and C most common in New Zealand (3)
- Wilson's Disease, a rare genetic disorder of copper accumulation in liver and brain
- Idiopathic, cryptogenic, or indeterminate (without obvious cause)

Liver transplantation is a significant clinical decision and candidates must have an irreversible disease for which medical and surgical therapies have been exhausted. Despite being a last resort in critical situations, paracetamol aetiology is the fourth leading cause of liver transplants, as summarised in Figure 1 (4). Routine laboratory tests can aid in the differential diagnosis of ALF, and therefore it is important for laboratories to provide rapid and accurate laboratory test results to help determine the cause and ascertain effective treatment without relying on a liver transplant.

Pathophysiology of paracetamol

Although paracetamol is widely used for pain relief, the mechanism of action is a topic of debate. It is known that paracetamol confers its analgesic effects by activating the descending serotonergic pathways, and Anderson (5) proposes two mechanisms: inhibition of prostaglandin (PG) synthesis or influencing cannabinoids receptors. In the inhibition pathway, prostaglandin H2 synthetase (PGHS) is responsible for arachidonic acid metabolism to the unstable PGH2. There are two active sites for PGH2: a cyclooxygenase (COX) and a peroxidase (POX) site. Paracetamol acts on the POX site and reduces the amount of the oxidised form of the COX site, thereby inhibiting the synthesis of PG. Alternatively, the synthesis can be inhibited by an active metabolite of paracetamol (p-aminophenol) and acts on the cannabinoid receptor, which exerts analgesic effects such as relaxation and tranquillity. At therapeutic doses, more than 90% of the paracetamol is metabolised by the liver and excreted in urine as nontoxic glucuronide and sulphate conjugates.

As schematically represented in Figure 2, less than 5% of paracetamol is metabolised by cytochrome P450 1AE and 2E1 to N-acetyl-p-benzoquinoeimine (NAPQI), which is a highly toxic intermediate that causes free radical damage. Moreover, the cytochromes can be induced to increase activity and thereby produce more NAPQI under cases of paracetamol overdose, alcohol consumption, or induction by isoniazid and phenobarbitones (6). This toxic intermediate is usually reduced to nontoxic mercapturic acid and cysteine conjugates, but in the case of paracetamol overdose, where sulfation and the glucordination pathway becomes saturated, glutathione becomes deficient and excessive NAPQI nonspecifically binds to intracellular proteins causing cell apoptosis and ultimately death.

A timely measurement paracetamol concentration allows treatment with N-acetylcysteine (NAC) that is known to minimise or prevent hepatic damage (7). The mechanism for NAC to be the antidote is not fully understood. It appears NAC is able to work as a sulfhydryl donor and replenish the hepatic glutathione storage and sulphate conjugation (8,9). However, the effectiveness of the drug rapidly diminishes 12 to 24 hours after exposure to paracetamol. This reinforces the importance of a rapid and accurate measurement of paracetamol to establish whether NAC treatment is beneficial. Therefore, plasma paracetamol concentrations should be measured as soon as possible for all cases of suspected drug overdose. Falsely positive paracetamol results can lead to inappropriate use of NAC. This can trigger potential serious adverse effects, where gastrointestinal effects are the most common. Most importantly, anaphylactoid reactions occur in around 60% of patients. In addition, clinicians may make false assumptions about the cause of the ALF and take no further investigation for other possible causes. The true cause may never be found, and patients may miss the needed treatment.

Methods of paracetamol measurement

Many methods have been described for the assay of paracetamol. The gold standard is Gas Liquid Chromatography (GLC) and High Pressure Liquid Chromatography (HPLC) as it

has proven to produce accurate quantification of paracetamol with minimal interference (1). Notwithstanding, these tests are time consuming, require specialised laboratory equipment, and highly skilled staff to operate the instrument making it unsuitable for routine laboratory measurement. For rapid, automated measurement of paracetamol colorimetric enzymatic methods are more commonly used. Another automated method is the competition immunoassay that is widely available but more expensive. In 2019, 117 on the 322 laboratories enrolled in the Royal College of Pathologists of Australasia's Quality Assurance Program (RCPAQAP) used methods with a colorimetric measurement. Incidentally, 69 of the laboratories used immunoassay. In our laboratory at LabPLUS, paracetamol is measured with Cobas ACET2 which is based on competition immunoassay (10).

Interference with paracetamol measurement

Colorimetric principles, whilst economical, are more susceptible to spectrophotometric interferences that immunoassays are less likely to experience. This includes haemolytic and icteric separated plasma that create a strong background interference in the absorbance of the ultraviolet and visible regions of the electromagnetic spectrum. In particular, icteric samples can raise the background absorbance at 600 nm that contributes to a false elevation in paracetamol. According to the package insert for an enzymatic principle (11) interference occurs at unconjugated bilirubin concentrations of >86 µmol/L. Moreover, enzymatic principles may also have a false positive in the presence of therapeutic concentrations of NAC (8). Unlike enzymatic based principles, immunoassays are less likely to have interference from bilirubin, up to 510µmol/L of unconjugated bilirubin, and unaffected by presence of NAC.

DISCUSSION

Because paracetamol levels were consistently elevated throughout the subsequent samples taken during hospitalisation, interference with the assay was considered to be an issue. Particularly, bilirubin interference is known to cause aberrant results in colourimetry. To reduce this interference, plasma ultrafiltration was applied to remove the interfering substances before measuring paracetamol with the enzymatic method. An ultrafiltrate has the same concentrations of analytes but is virtually free of proteins, including protein-bound bilirubin, haemoglobin, and lipoproteins. Using Centrifree micropartition devices from Amicon Bioseparations/Milipore, 1 mL of patient plasma and positive control in the reservoir of the device was centrifuged at 1000-2000g for 10 min. The ultrafiltrate was collected, assayed, and quantified for paracetamol and bilirubin with the Vitros system.

The results summarised in Table 2 indicated that the patient had not ingested paracetamol, as the analyte was undetectable in the patient's plasma by the enzymatic method assay post-treatment. These findings were supported by the HPLC method that consistently produced negative results on the patient's plasma samples. Interpreted together, the results strongly indicate bilirubin interference and false positive paracetamol concentrations when measured by the enzymatic method. An alternative method for identifying bilirubin interference for laboratories that do not have the ultrafiltration equipment is dilutions of the sample to confirm the bilirubin effect.

From previous research, the colorimetric method on the Vitros system was the only method that expressed positively proportional linear relationship between the false positive value of paracetamol and bilirubin concentration (12). Among other colorimetric methods, the Vitros system is the only one using ferricyanide as chromogen activator. It is possible that ferricyanide reacts with bilirubin or unknown substances in the sample to produce product that absorbs at or around the wavelength λ=670 nm. This concept was supported when normal human serum with added bilirubin was tested. Since conjugated and unconjugated bilirubin have an absorption peak between 390 and 460 nm it is therefore likely that bilirubin by-products are causing interference in the assay.

The patient's liver functions improved during hospitalisation, and she made an uneventful full recovery after three weeks. The exact pathophysiology of acute hepatic derangement remains obscure, as Fong *et al.* conclude (1). Possible explanations of over-the-counter health supplements containing hepatotoxic ingredients, such as alternative herbal remedies or a rare viral hepatitis caused by the Epstein-Barr virus were suggested, but not rigorously excluded in this case.

CONCLUSIONS

Persistently increased levels of paracetamol were seen in a patient with acute liver failure which was initially suspected due to the inability to metabolise the drug, despite patient describing they had not taken the drug. Timely laboratory results of paracetamol measurement are critical in the management of overdose, as prompt treatment using glutathione precursor NAC can minimise hepatic damage, but its effectiveness diminishes rapidly by 12-24 hours after exposure. Many methods of paracetamol analysis have been described and the colorimetric enzymatic assays are commonly used for its rapid and economic automated protocol. Despite these benefits, the colorimetric principle is more susceptible to spectrophotometric interference from bilirubin and is used in more than a third of the laboratories enrolled in RCPAQAP in Australasia. Particularly in the case of acute liver failure, high total bilirubin can give rise to false positive paracetamol levels, confounding the clinical picture. It is highly recommended that total bilirubin is analysed for all paracetamol analysis, particularly if the laboratory is using a colorimetric method.

Table 1. Initial presentation of laboratory results.

Test	Results	Units	Reference range
Total Bilirubin	1210	µmol/L	<25
AST	5080	U/L	0-45
ALT	6170	U/L	0-45
ALP	150	U/L	40-110
Ammonia	171	µmol/L	<70
LDH	6830	U/L	120-250
Paracetamol	121	µmol/L	<100
PT	39.7	seconds	9-18
INR	3.3	-	2-3
Hepatitis A Serology	Negative	-	-
Hepatitis B Serology	Negative	-	-

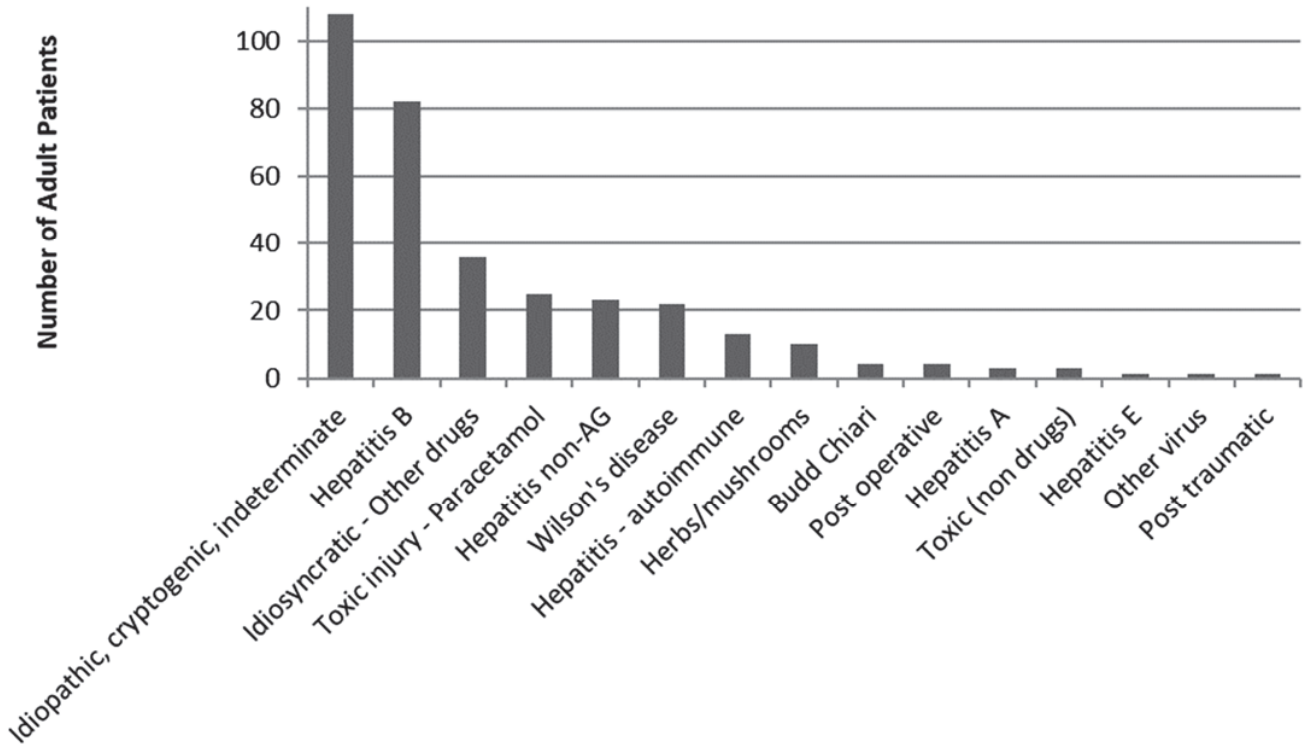

Figure 1. Data of adult acute liver transplant cases in Australia and New Zealand to 31/12/2019. Adapted from the Australia and New Zealand Liver and Intestinal Transplant Registry 31st Annual Report 2019.

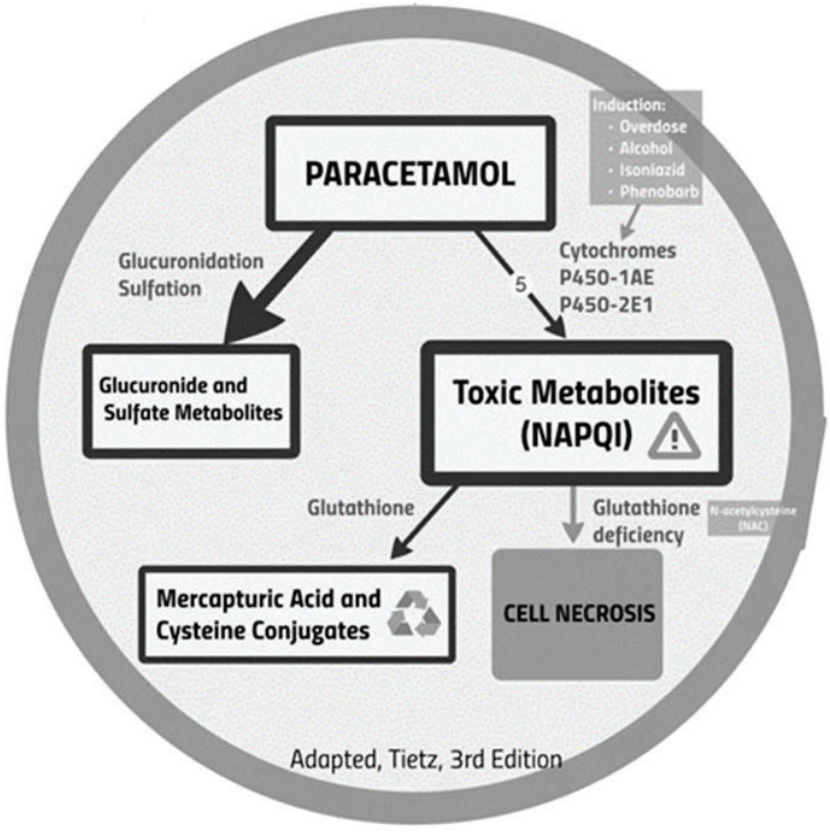

Figure 2. Paracetamol pathophysiology (adapted from Tietz, 3rd Edition, 1999).

Table 2. Paracetamol and bilirubin concentrations for patient samples day 1-3.

Method (Day 1 sample)	Test	Patient (µmol/L)	Positive control (µmol/L)
Plasma, enzymatic	Paracetamol	121	610
	Bilirubin	1210	12
Ultrafiltrate, enzymatic	Paracetamol	<30	609
	Bilirubin	<2	<2
Plasma, HPLC	Paracetamol	<1	612

Method (Day 2 sample)	Test	Patient (µmol/L)	Positive control (µmol/L)
Plasma, enzymatic	Paracetamol	115	611
	Bilirubin	1170	12
Ultrafiltrate, enzymatic	Paracetamol	<30	610
	Bilirubin	<2	<2
Plasma, HPLC	Paracetamol	<1	613

Method (Day 3 sample)	Test	Patient (µmol/L)	Positive control (µmol/L)
Plasma, enzymatic	Paracetamol	104	609
	Bilirubin	960	13
Ultrafiltrate, enzymatic	Paracetamol	<30	607
	Bilirubin	<2	<2
Plasma, HPLC	Paracetamol	<1	612

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