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A MODIFIED LOW COST COLOURIMETRIC METHOD FOR PARACETAMOL (ACETAMINOPHEN) MEASUREMENT IN PLASMA

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Abstract

Background—Despite a significant increase in the number of patients with paracetamol poisoning in the developing world, plasma paracetamol assays are not widely available. The purpose of this study was to assess a low cost modified colorimetric paracetamol assay that has the potential to be performed in small laboratories with restricted resources.

Methods—The paracetamol assay used in this study was based on the Glynn and Kendal colorimetric method with a few modifications in order to decrease the production of nitrous gas and thereby reduce infrastructure costs. Preliminary validation studies were performed using spiked aqueous samples with known concentrations of paracetamol. Subsequently, the results from the colorimetric method for 114 stored clinical samples from patients with paracetamol poisoning were compared with those from the current gold-standard high-performance liquid chromatography (HPLC) method. A prospective survey, assessing the clinical use of the paracetamol assay, was performed on all patients with paracetamol poisoning attending the Peradeniya General Hospital, Sri Lanka over a ten-month period.

Results—The recovery study showed an excellent correlation ($r^2 > 0.998$) for paracetamol concentrations from 25–400 mg/l. The final yellow colour was stable for at least 10 minutes at room temperature. There was also excellent correlation with the HPLC method ($r^2 = 0.9758$). In the clinical cohort study, use of the antidote N-acetylcysteine was avoided in over a third of patients who had a plasma paracetamol concentration measured. The cost of consumables used per assay was \$0.50 (US).

Conclusions—This colorimetric paracetamol assay is reliable and accurate and can be performed rapidly, easily and economically. Use of this assay in resource poor clinical settings has the potential to have a significant clinical and economic impact on the management of paracetamol poisoning.

Keywords

Paracetamol; Acetaminophen; paracetamol concentration; hepatotoxicity; overdose

Introduction

Paracetamol is one of the most widely used analgesics/antipyretics worldwide. In patients presenting with paracetamol overdose, the current standard of care is to undertake a risk assessment based on measurement of the plasma paracetamol concentration and to compare this with a nomogram to establish the need for antidote treatment with N-acetylcysteine (NAC).¹ In some circumstances, such as the absence of an assay service, risk assessment can be made on the history of the ingested dose; however, this is generally leads to overtreatment of many patients who are at low risk of hepatotoxicity as there is a poor correlation between patients stated ingested dose of paracetamol and paracetamol concentration.^{2,3}

In Sri Lanka, the incidence of paracetamol poisoning gradually increased over five years (2004 - 2008) from 2.8% to 6.4% (95% CI 2.3 to 4.9; $P < 0.001$) (Unpublished data) Although pesticide poisoning remains more common, paracetamol is now the most common drug in patients presenting with self-poisoning. Paracetamol poisoning is becoming more common in other areas of South Asia such as Nepal.^{4,5} In Sri Lanka, and other areas of South Asia, laboratory facilities to determine plasma paracetamol concentrations are only available in a few private laboratories. As paracetamol assays are not widely available, risk assessment in patients presenting with paracetamol poisoning is based on the history of the dose ingested and these results in a significant proportion of individuals having unnecessary treatment with the antidote.^{6,7} The plasma paracetamol concentration is also important in patients admitted with paracetamol poisoning with an uncertain history and/or in individuals who have co-ingested agents that cause drowsiness.¹

The aim of this study was to assess and validate a low cost colorimetric paracetamol assay that has the potential to be performed in small laboratories with restricted resources and to look at its impact on the management of a cohort of patients with paracetamol poisoning.

Materials and Method

Reagents

Tri chloro acetic acid ($C_2HCl_3O_2$, MW 163.39), Sodium nitrite ($NaNO_2$, MW 69.0), Sodium hydroxide ($NaOH$, MW 40.0), Sulphamic acid (NH_2SO_3H , MW 97.09) and Hydrochloric acid (HCl , MW 36.46) were purchased from Sigma Chemicals. All reagents were of analytical (AR) grade. Paracetamol powder was used to prepare 1000 mg /L stock solution which was prepared by dissolving powder in warm distilled water. The stock solution was used to prepare eight series of (25 mg/L – 500 mg/L) working standards, 25, 50, 100, 150, 200, 250, 300, 350, 450 g/dl.

1. The colorimetric assay—The colorimetric method used was based on the Glynn and Kendal method with a few modifications (table 1) to the volume of sample and reagents in order to decrease the production of nitrous gas.⁸ In the modified method, 0.5 ml of plasma was pipetted into a 15 ml centrifuge tube containing 1.0 ml of 15% trichloroacetic acid. After vortex mixing, it was centrifuged briefly for three minutes and the clear supernatant was decanted into a 10 ml test tube containing 0.5 ml 6N hydrochloric acid. Nitrous acid was generated, by adding 0.4 ml of sodium nitrite to the resulted solution. After allowing the contents to stand for two minutes, 1.0 ml of 15% sulphamic acid was added carefully to

neutralize excess nitrous acid. Finally 2.5 ml of 15% sodium hydroxide was added and the absorbance of each sample was measured at 430 nm, against a reagent blank of water.

2. The validation experiments—A calibration curve was calculated using this method to measure standard paracetamol concentrations (25, 50, 100, 150, 200, 250, 300 and 400 mg/l using a UV-Visible spectrophotometer (Unico 2802). Within-run precision (CV) was evaluated by 14 different assays of paracetamol standards. The stability of the final coloured solution was also assessed up to 10 min by measuring the absorbance (this was an end point measurement rather than a kinetic one).

3. Comparison of the Colorimetric Assay with HPLC—The plasma samples used in this part of the study were taken from 114 patients with paracetamol poisoning admitted to Galle and Peradeniya Hospitals in Sri Lanka. The samples were analyzed using the colorimetric assay and were then stored at -20°C and analyzed using a reference HPLC method (Roche modular system -Roche Diagnostics, Mannheim, Germany) at Guy's and St Thomas' NHS Foundation Trust in London, UK.

4. Clinical study—A prospective survey was undertaken of all patients with paracetamol poisoning presenting to the Peradeniya General Hospital, Sri Lanka over a ten-month period from March 2008 to January 2009. Ethical approval for this and the above studies was obtained from the Ethical Review Committee, University of Peradeniya, Sri Lanka. Patients with suspected paracetamol poisoning had a sample taken for a plasma concentration at the time of admission. Paracetamol levels were measured during the daytime only. Doctors were asked to use the plasma paracetamol concentration, the time since ingestion and the consensus nomogram used in Australia and New Zealand to guide treatment.¹ When the plasma paracetamol assay was not available; commonly when the patient presented at night-time, an ingested dose of greater than 200mg/kg was used as the treatment threshold and if the paracetamol concentration was subsequently found to be below the risk level the NAC infusion was ceased.

Results

The colorimetric assay paracetamol assay validation studies in aqueous solution were found to perform well up to a concentration of 400 mg/L ($r^2=0.998$); figure 1 shows the calibration plot for the standard paracetamol solutions and correlation coefficient for the calibration curve. The coefficient variant and accuracy for inter batch standards were 1.1% and 0.21% respectively. The change in the absorbance of the final solutions per minute was constant up to 10 minutes for all standards.

There was excellent correlation between the paracetamol concentration in the stored plasma samples by HPLC and colorimetric method ($r^2 = 0.98$) (figure 2). The Bland Altman plot for paracetamol concentrations estimated by the colorimetric method and HPLC is shown in figure 3.

A total of 99 (10 patients per month) paracetamol-poisoning patients were identified, of whom 74 had a paracetamol concentration measured and 43 patients were treated with NAC (figure 4). The measurement of a plasma paracetamol concentration was used to identify 27 (36.5 % of those who had a paracetamol assay) patients who did not require NAC.

Discussion

Paracetamol poisoning results in only non-specific clinical features such as nausea and vomiting in the early stage of poisoning and the risk of liver damage is normally predicted

from the plasma paracetamol concentration.^{1 2} Therefore the estimation of plasma paracetamol concentrations is important in all patients with paracetamol poisoning to identify the severity of poisoning, the need for treatment with the antidote NAC and to guide prognosis.^{1 9} HPLC paracetamol assays are not widely available in most hospitals in Sri Lanka and South Asia and therefore risk assessment in this patient group can be problematic.⁶

A number of assay techniques have been assessed for paracetamol; these techniques have variable costs and different requirements for technical skill and equipment. These include colorimetric techniques,^{10 11 8} UV spectrophotometric methods,¹² immunoassays,¹³ high performance liquid chromatography (HPLC),^{14 15} Gas Chromatography (GC),^{16 17} and electrophoresis.¹⁸ HPLC is generally accepted as the method of choice. However, both HPLC and GC are expensive techniques with expensive equipment and high operational and maintenance costs. Some enzymatic –colourimetric assays give false results in paracetamol levels, which may be due to methodological interferences of bilirubin.^{19 20} The method of Glynn and Kendal is little affected by paracetamol conjugates and gives more reliable measure of unchanged (free) drug. Previous research reports that interference by salicylates and salicylamide can occur.^{21 22} Interference by salicylates, although slight, can be corrected using modifications in the method.²³ Furthermore, amitriptyline, amphetamine, caffeine, chlordiazepoxide, chlormezanone, chlorpromazine, chlorpropamide, cocaine, diazepam, diphenhydramine, dihydrocodeine, imipramine, indomethacin, lorazepam, meprobamate, methadone, methaqualone, morphine, nitrazepam, oxypertine, pentazocine, pentobarbital, phenacetin, phenytoin, promethazine, strychnine, theophylline, and tolbutamide reportedly do not interfere with this method. There is no interference by NAC with method.²⁴

The cost per test using an immunoassay method is high due to cost of kits. The colorimetric assay method is less expensive but it is not currently available in Sri Lanka or other areas of South Asia. In general, the cost per test using HPLC is US\$ 124 compared to less than US\$ 1 per test for a colorimetric assay. Furthermore, colorimetric techniques are more applicable to the instrumentation available and testing frequency of small hospitals and rural treatment centers in South Asia, due to minimal reagent cost and waste.²⁵ Despite this they have not been implemented, barriers have included local validation, training and a requirement to vent noxious gases.

We have shown that this new colorimetric assay for paracetamol is accurate, both in aqueous solution and more importantly in plasma samples from patients with paracetamol poisoning over a clinically relevant range of paracetamol concentrations. The modifications that we have introduced to the method allowed the assay to be performed without the need for a fume hood, using laboratory consumables and equipment that is widely available in Sri Lanka and other areas of South Asia. The instrument needed for the test is a spectrophotometer, which is available in many hospitals in South Asia. The instrument cost is approximately US\$4,000-8,000 and the reagent cost per test is about US\$ 0.50.

In the clinical cohort study, use of the antidote NAC was avoided in over a third of patients who had a plasma paracetamol concentration measured; the availability of the colorimetric assay therefore had a significant positive impact on the management of this group of patients. Furthermore, this has the potential to have a significant economic impact. We have shown that in Sri Lanka and other areas of the developing world, the cost of NAC is the major cost in patients with paracetamol poisoning.⁴ Previous studies have shown that the introduction of an appropriately used, accurate and cost-effective paracetamol assay was a central strategy in reducing costs and improving the standard of care of these patients.⁴ Most hospitals currently have the facilities to be able to perform the colorimetric assay; therefore

the additional costs of US\$ 16 (31 patients) in performing paracetamol assays would result in up to four patients avoiding unnecessary treatment with NAC and a cost saving of US\$ 576 (Figure 4).

In countries in which paracetamol poisoning is prevalent and the cost of antidote is a high component of total cost introduction of a paracetamol assay service appears cost effective and may reduce total expenditure.

Conclusion

This new colorimetric paracetamol assay is reliable accurate and can be performed rapidly, easily and economically. Its introduction into Sri Lanka and hospitals in other areas of South Asia and the developing world has the potential to have a significant impact from both a clinical and economic perspective. This would result in an improvement in the management of an increasing clinical problem in light of the changing epidemiology of poisoning in Sri Lanka and other areas of South Asia.

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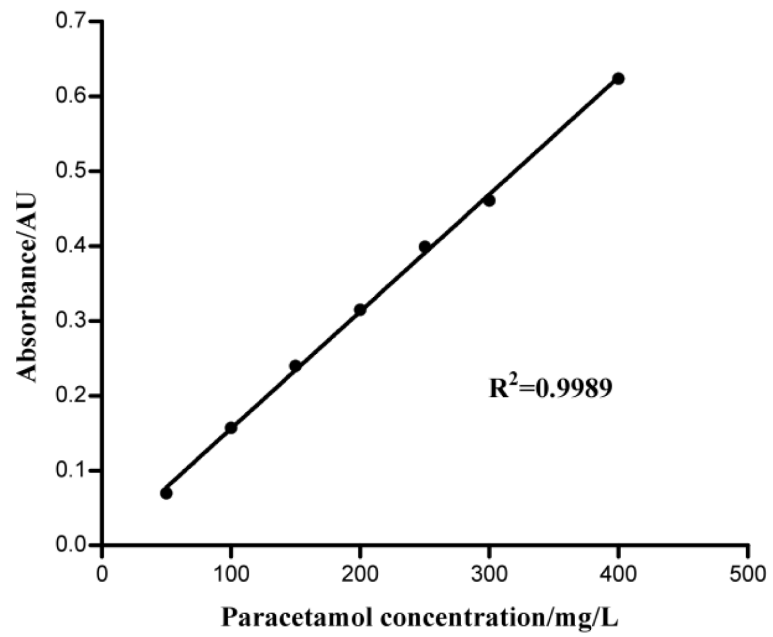


Figure 1.
An example of calibration plot of standard paracetamol samples at wavelength of 430 nm.

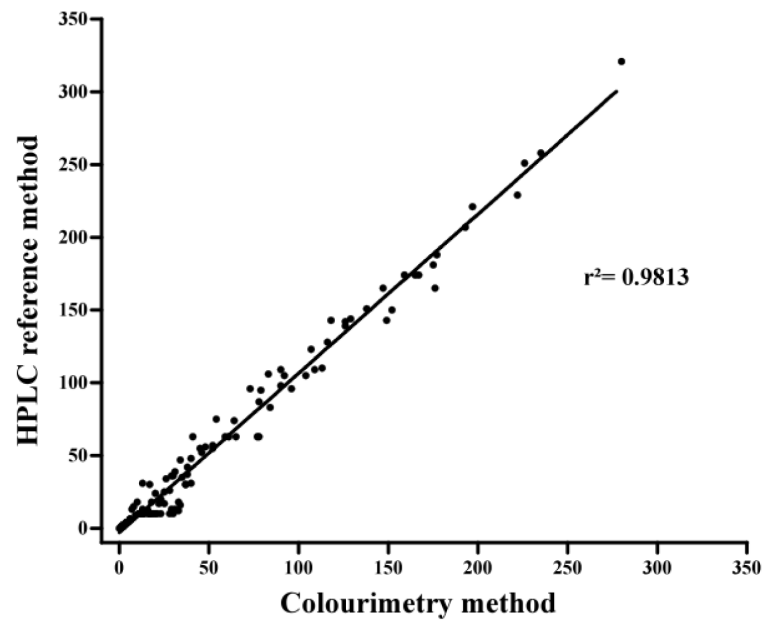


Figure 2. Correlation of results for plasma paracetamol concentration measured by the present method and with HPLC reference method

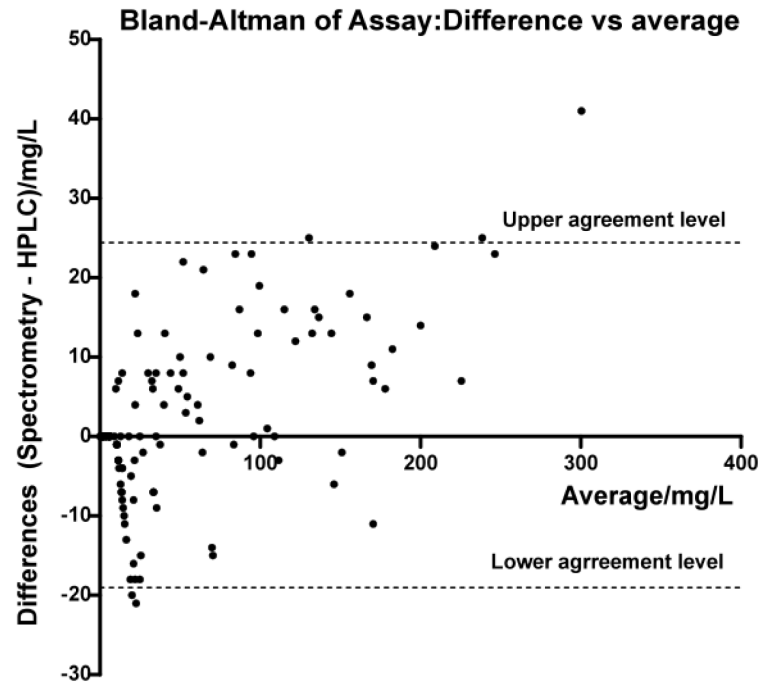


Figure 3.
Bland and Altman plot for paracetamol measured by present method and HPLC method.

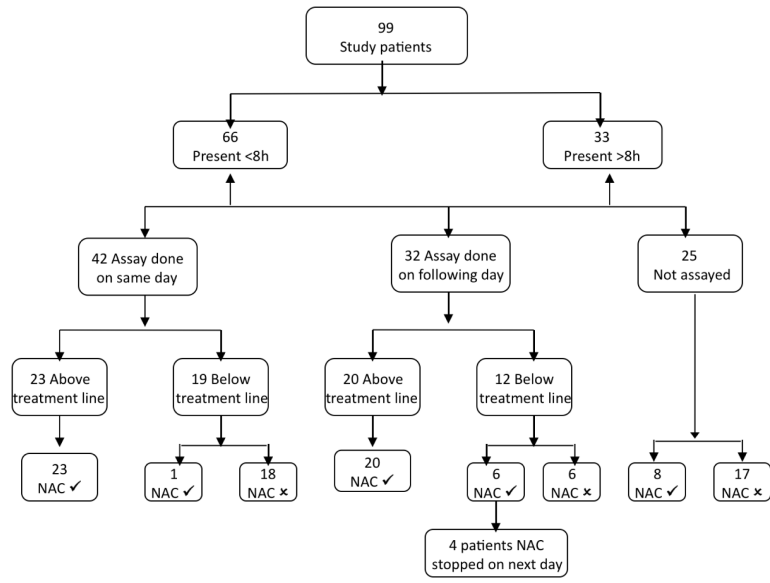


Figure 4. Patient’s treatment results based on the paracetamol levels.

Table 1

Modification of Glynn and Kendal colourimetric method.

	Glynn and Kendal colorimetric method	Modification in Proposed method
Amount of sample	1.00 ml	0.5 ml
Trichloroacetic acid	2.00 ml	1.00 ml
6N hydrochloric acid	0.50 ml	0.5 ml
Sodium nitrite	1.00 ml	0.40 ml
Sulphamic acid	2.00 ml	1.00 ml
Sodium hydroxide	5.00 ml	2.50 ml