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Journal of Food and Drug Analysis, Vol. 16, No. 2, 2008, Pages 36-40 藥物食品分析 第十六卷 第二期

Novel Spectrophotometric Method for RAPID Quantifying Acetaminophen Concentration in Emergent Situation

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(Received: May 8, 2007; Accepted: September 27, 2007)

ABSTRACT

A novel spectrophotometric method for rapid quantification of acetaminophen in serum that is particularly suited for emergency usage is described. Free unconjugated acetaminophen is separated from other endogenous interferents by extracting the drug into ethyl acetate by simply using a vortex mixer. Subsequently, the cupric ions in the reagent are reduced by the phenolic hydroxyl groups present in the drug. The resultant cuprous ions then interacted with bicinchoninic acid (BCA) to form a chromophore which absorbs maximally at 562 nm. The proposed method has a linearity range from 50 to 400 μg/mL. The method was precise with day-to-day coefficients of variation (CVs) for two controls (44 and 195 μg/mL) of 5.0 and 4.5%, respectively. Results obtained by the proposed method correlated excellently with those determined by either an established HPLC or a Schiff's base dye formation method with correlation coefficients of 0.98 and 0.99, respectively. A group of commonly used prescription drugs or compounds of potential interferents were tested and found not to interfere. The proposed method for acetaminophen determination, which has a turnaround time of <10 min, is simple and rapid. For this reason, it is especially suitable for the screening of drug overdose in an emergency situation.

Key words: acetaminophen, cuprous/bicinchoninic acid complex, overdose, hepatotoxic effect

INTRODUCTION

Acetaminophen (Tylenol; paracetamol; APAP) is a popular analgesic and antipyretic medication which has been promoted as a safe alternative to aspirin. Despite certain aspirin side effects are less prominent with APAP such as gastric irritation and effects on blood clotting, the drug is not innocuous. With the increased usage of APAP, both accidental excessive ingestion of the drug in children and intentional ingestion in adolescents have increased rapidly. After ingestion of an overdose amount of APAP, fatal centrilobular hepatic necrosis and acute tubular necrosis are the ominous complications which have been documented to be due to the accumulation of toxic metabolites of this drug[1-6].

Proper diagnosis and treatment of APAP overdose requires the determination of APAP blood levels obtained 4 hr or more after ingestion. This information, along with the time of ingestion should enable the clinician to determine the probability of potential hepatotoxic reaction. However, the information may sometimes be difficult to obtain if the patient is in a comatose state. Thus, it is imperative that the clinical laboratory of a hospital with emergency services could perform fast, accurate and reliable analyses for serum or plasma APAP so that the clinician can treat APAP overdose cases in a minimum amount of time.

They are many reliable laboratory methods for quantifying serum APAP levels, but they are often time-consuming, techniquically demanding and require the use of costly, highly specialized instruments making them unsuitable for the emergency usages[7-10]. In addition, several colorimetric methods have also been devised for assaying APAP. However, several of these methods measure inactive conjugates along with free APAP and salicylate interferes with the most popular colorimetric methods[11,12]. For these reasons, we have specifically developed a rapid and facile assay method for APAP in serum in order to meet the emergency need.

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Journal of Food and Drug Analysis, Vol. 16, No. 2, 2008

The proposed method is based on the reduction of cupric ions by the phenolic hydroxyl groups of the drug and the subsequent formation of a colored chromophore between the resultant cuprous ions and bicinchoninic acid (BCA) that can be measured spectrophotometrically. This proposed method is well suited for an emergency purpose because of its short turnaround time (<10 min).

MATERIALS AND METHODS

I. *Chemicals and Reagents*

All chemicals used were of analytical grade and doubly distilled water was used throughout. Micro BCA protein assay method, which was previously reported by Smith *et al.*[13], was adopted for this study. The kit could be obtained commercially (Pierce Chemical Co., Rockford, IL, USA). The commercial prepared kit contained three separated bottles, namely: (1) Micro reagent A (MA) containing sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH; (2) Micro reagent B (MB) containing 4% BCA in H_2O; and (3) Micro reagent C (MC) containing 4% cupric sulfate, pentahydrate in H_2O. These reagents are stable for at least 12 months when stored at room temperature.

The working BCA reagent was prepared by mixing 2 parts of MC and 48 parts of MB, followed by adding 50 parts of MA and this working solution is stable for one day at room temperature.

II. *Procedure*

Standard, control or unknown (0.5 mL) was pipetted into a 10 mm × 75 mm glass tube. One milliliter of ethyl acetate and a few crystals of NaCl were added in a chemical hood. The mixture was mixed vigorously for 30 sec and let stand for a few seconds for the phase separation. The upper organic layer (0.1 mL) was removed and added to a pre-prepared test tube containing 2.0 mL of working BCA solution. The reaction mixture was mixed gently and let stand at room temperature for exactly 5 min. A reagent blank was also prepared. The absorbances at 562 nm of all tubes were measured. The concentration of APAP can then be calculated using the following formula:

$$\frac{A_{unknown} - A_{reagent\ blank}}{A_{standard} - A_{reagent\ blank}} \times \text{conc of standard } (\mu g/mL) = \text{APAP } (\mu g/mL)$$

RESULTS

I. *Linearity*

As shown in Figure 1, our proposed method for

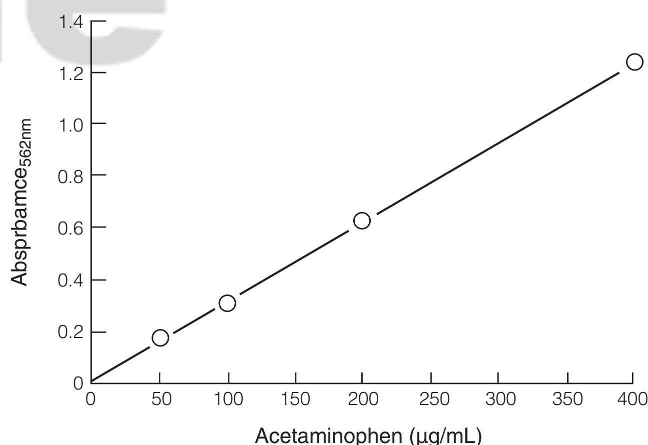

Figure 1. Relation between acetaminophen concentration and absorbance at 562 nm. Each point represents an average of triplicate determinations.

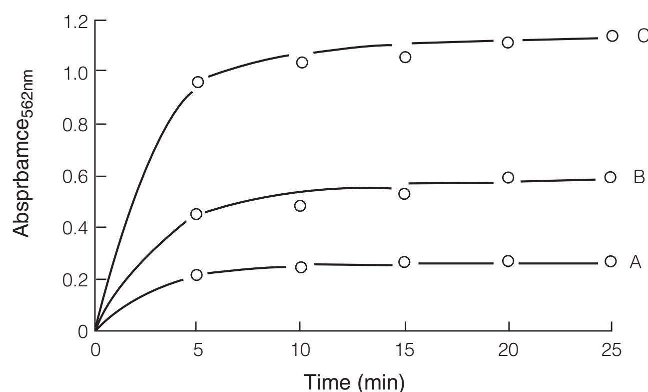

Figure 2. Color stability of reaction product acetaminophen concentration. (A) 50 μg/mL; (B) 100 μg/mL; (C) 200 μg/mL.

APAP has a linear range from 50 to 400 μg/mL. This range is sufficient to cover both the non-toxic and toxic levels of APAP.

II. *Color Stability*

The time course study for color stability was performed using three levels of APAP versus incubation time. As shown in Figure 2, the net absorbances of these reaction mixtures reach nearly plateaus after 5 min of reaction between drug-reduced cuprous ions and BCA.

III. *Precision Studies*

Reproducibility as reflected by the within-run and the day-to-day precision data was good (Table 1). Six repetitive determinations on two pooled APAP-spiked serum controls had mean values of 44.0 and 194 μg/mL with coefficients of variation (CVs) of 7.3 and 4.7% respectively. The CVs for the same set of controls

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Table 1. Precision studies results

	Control I	Control II
Within-run (n = 6)		
Mean (μg/mL)	44.3	194.5
SD (μg/mL)2	3.2	9.2
CV (%)	7.3	4.7
Day-to-day (n = 42)		
Mean (μg/mL)	46.5	96.3
SD (μg/mL)	2.3	8.8
CV (%)	5.0	4.5

assayed on 7 consecutive days were 5.0 and 4.5%, respectively. Again, the day-to-day reproducibility was acceptable.

III. *Analytical Recovery*

The percentage recovery represents the measured value expressed as a percentage of the expected value. The mean percentage recovery for 10 samples was 98% with a standard deviation of 4.3% (data not shown).

IV. *Correlation Studies*

We compared results of APAP obtained by the proposed method with those determined by the established HPLC method[7] for 23 samples (Figure 3A). A correlation coefficient of 0.98 was obtained. The regression line was y = 1.07 × (-4.9). In this study, the y-intercept was 4.9 μg/mL, meaning that a constant error of 4.9 μg/mL occurred. The slope was 1.07, indicating a proportional error of -7.0%. Also, the results of APAP assayed by the proposed method were compared to those measured by the Schiff's base dye formation method[14] for 24 samples (Figure 3B) and a correlation coefficient of 0.99 was obtained. The regression equation was y = 1.03 × (-4.3). In this study, the y-intercept was 4.3 μg/mL. The slope was 1.03, meaning a proportional error of -3% was observed.

V. *Interference Studies*

A group of drugs and compounds were tested for their potential interferences on the proposed method for APAP. The drugs which exerted no interference (100 mg/mL) (<3% change) were: phenylephrine, phenylbutazone, caffeine, theophylline, ascorbic acid, phenacetin, acetylsalicyclic acid, diazepam, digoxin, quinidine and phenytoin.

DISCUSSION

A variety of spectrophotometric methods have previously been devised for quantifying APAP in serum of plasma based on the unique functional groups attached on the aromatic nucleus of this drug[14,15]. APAP, a N-acetylated p-aminophenol, was capable of being deacetylated by heat and acid to yield p-aminophenol (PAP). The primary amine present in PAP can thus be used to generate either colorful chromopheres via Schiff's base formation through interacting with aldehydes (e.g., p-dimethylaminocinnamaldehyde) or an indophenol derivative by oxidative coupling with a phenolic compound (e.g.,

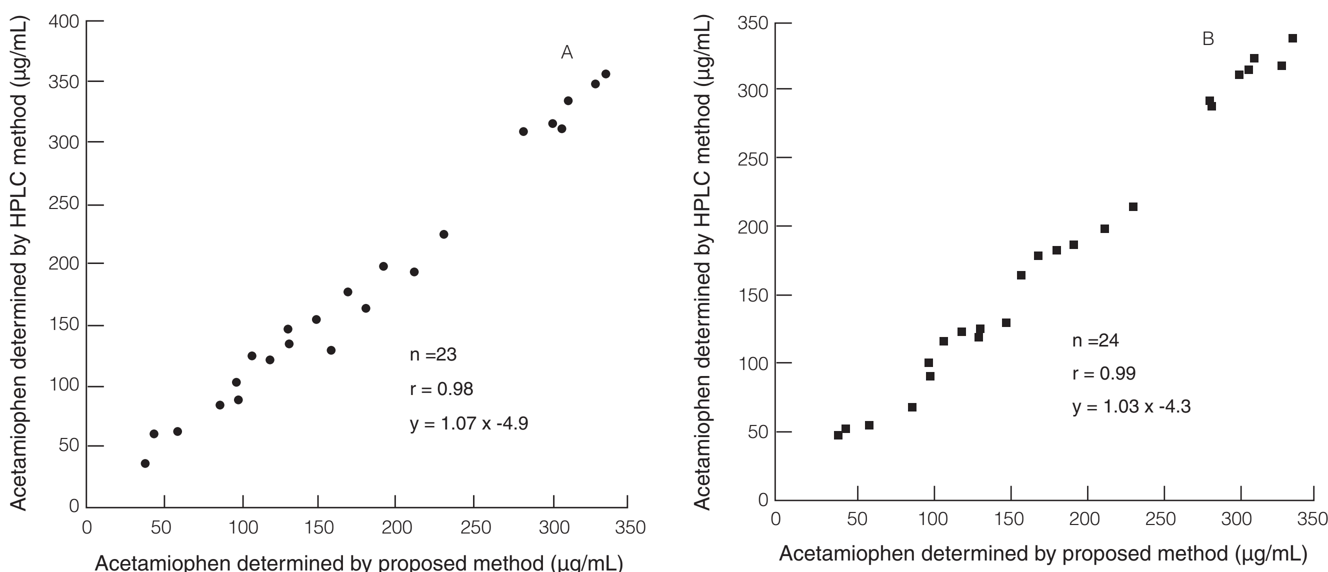

Figure 3. Correlation of results for acetaminophen concentration as obtained by the proposed method and with (A) the HPLC method or (B) the Schiff's base dye formation method.

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p-xylenol). These methods, though specific, are rather cumbersome owing to the necessity of a lengthy deacetylation step in order to generate PAP. In addition, improper control of the hydrolysis temperature can lead to inaccurate results. Alternatively, phenolic hydroxyl group present in APAP has been found to be capable of reducing ferric 2,4,6-tris (2-pyridyl)-s-triazine (Fe^{3+}-TPTZ) complex, at acidic PH, to its ferrous-TPTZ counterpart which absorbs maximally at 593 nm. This observed phenomenon is referred as the direct ferric reduction (DAFR)[16] and has been the chemical basis for another method for quantifying APAP in serum and plasma[17]. The minor shortcoming of this method was that the increment of absorbances would proceed with time and reached plateau in approximately 30 min. The time of reaction and measurement of each specimen should thus be exactly timed so that the occurrence of erroneous result can be obviated.

Smith *et al.*[13] reported the use of BCA as a protein assay reagent with a minimum detection level of just 0.5 μg/mL for BSA. The principle of the assay is based upon the detection of cuprous ion (Cu^{+}), which is formed when cupric ion (Cu^{2+}) is reduced by protein in an alkaline environment. The purple-colored product is formed by the chelation of two molecules of BCA to the cuprous ion, which exhibit a strong absorbance at 562 nm. Further studies indicated that the number of peptide bonds, and the presence of four amino acids (cysteine, cystine, tryptophan, and tyrosine) are responsible for color formation with BCA[18]. In addition, some biologically relevant compounds, such as catecholamines and uric acid have been reported to be the interferents of BCA assay for protein determination[19]. Based on these observations, it can be speculated that APAP, a structural analog of catecholamines, can also reduce Cu^{2+} to generate Cu^{+} which will ultimately be chelated with BCA molecules to form a colored chromphore. This speculation turns out to be true. We demonstrated here that APAP could generate an identical colorful chromophore in analgous to that of protein when reacting with a commercially available working BCA. The linearity of the assay ranged from 25 up to 400 μg/mL, which is sufficient to cover normal as well as overdose cases. Moreover, we also demonstrated that the interaction between APAP and working BCA reagent is fast and merely 5 min is sufficed to reach the platean (Figure 2). This characteristic along with the quick extraction of 30 sec with ethyl acetate to obviate nonspecific reductants present in the serum makes this method for quantifying APAP the simplest of all the procedures available to date. The turnaround time of a single determination is less than 10 min. Only a standard spectrophotometer is required. In addition, an added advantage of this assay for APAP is that the needed reagents have been standardized and are available commercially. All these reagents can be stored at room temperature and stable for at least one year. For this reason, in the event that an emergent APAP assay is requested by a physician, the laboratory technician can quickly assemble the working BCA solution without much delay.

In summary, our proposed assay provides a rapid, simple method for quantitative determination of APAP in serum or plasma. The method is especially suitable for an emergency situation because of its short turnaround time (<10 min).

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