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Modification of the Syva Emit® st™ Urine Amphetamine Assay

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ABSTRACT

The Syva Emit® st™ Urine Amphetamine Assay qualitatively determines the presence of urine amphetamine, one sample at a time. In each test, one sample and one calibrator are always assayed together to compare the reaction rates. This method is inefficient and expensive. Detailed analysis of the reaction kinetics of the Emit® st™ Urine Amphetamine reagents indicated that the reaction kinetics were relatively linear up to 15 minutes after the initiation of the reactions. This makes it possible to assay multiple samples with a single calibrator. A modified protocol is described which analyzed three samples with each calibrator, a modification leading to savings of one -third of the reagents and cost, with no compromise in assay performance. Addition of an extra 2 ml of distilled water to the regular protocol can lead to even more savings.

Key words : Emit®, Amphetamine, Cost effective.

INTRODUCTION

Immunoassays are the method of choice mandated by the United States Government for "drugs of abuse" screening program[1]. The Syva Emit® (Emzyme Multiplied Immunoassay Technique) reagents for drugs of abuse are widely used for the initial screening of drugs in urine samples[2]. The Emit® st™ Amphetamine Assay is a homogeneous immunoassay for use in qualitative analysis of amphetamines in human urine[3]. Emit® reagents were designated as the d.a.u.™ or st™ for different types of instrument . The d.a.u.™ assays can be adapted to most clinical chemistry analyzers in hospitals[4-9], while the st™ assays are designed for the Drug Detection System manufactured by the Syva Company. The Emit® st™ Amphetamine Assay is one of the reagents currently used by many local health authorities in Taiwan (including this laboratory) to screen urine amphetamine and/or methamphetamine. This Assay does not require expensive instruments and is easy to perform; results can be obtained in about two minutes by personnel who do not require extensive training [10]. An experienced operator can do 30 samples in one hour. However, the reagents are fairly expensive (260 NT dollars per sample). With the explosion of drug abuse problems in Taiwan and the rapid increase in samples to be screened in recent years[11], costs to society can become prohibitive.

This report describes a detailed analysis of

the reaction kinetics of the Emit® st™ Urine Amphetamine Assay, and recommends a modified procedure to achieve a saving of one-third of the reagents without affecting the assay performance.

MATERIALS AND METHODS

I. *Principles*

The Emit® assay is a homogeneous enzyme immunoassay technique used for the analysis of specific compounds in biological fluids[2]. The assay is based on the competition between drug in the sample and drug labeled with the enzyme glucose-6-phosphate dehydrogenase (G6P-DH) for antibody binding sites. Enzyme activity decreases upon binding to the antibody, so the drug concentration in the sample can be measured in terms of enzyme activity. Active enzyme converts oxidized nicotinamide adenine dinucleotide (NAD) to the reduced form (NADH), resultng in an absorbance change that is measured spectrophotometrically[3].

The Emit® st™ reagents are supplied in glass vials, each of which contains dry powder mixtures of antibody, enzyme, coenzyme NAD and substrate glucose-6-phosphate (G6P). The addition of 3 ml of water and 50 μl of the calibrator or samples starts the reaction. The reaction rates of the calibrator and the sample are determined by inserting the two vials into the Emit® st™ Drug Detection System to measure the absorbance at 340 nm. The reaction rates are automatically calculated by the instrument, with results printed out in 1.5 minute. This is a qualitative assay. The rate of reaction from the sample is compared to that from the calibrator, with a result expressed as either positive or negative [10].

II. *Samples*

Urine samples submitted for amphetamine screening assay by the criminal justice system in Hualien County were first assayed with the regular protocol, aliquoted and frozen at -20 degree C. Samples were thawed just before use.

III. *Reagnts*

Syva Emit® st™ Urine Amphetamine Assay reagents and calibrators were obtained from the local distributors of the Syva company.

IV. *Emit® st™ Urine Amphetamine Assay*

The presence of amphetamines in urine was screened by the protocol recommended by the manufacturer[3]. Briefly, the calibrator or sample at 50 μl and 3 ml of distilled water were added to the reagents vials, mixed well and the reaction rates determined with a Syva st™ Drug Detection System[10]. In the modified protocol, an additional 2 ml of distilled water was added, and reaction rates were monitored similarly.

V. *Reaction Kinetics of the Emit® st™ Urine Amphetamine Reagent*

Because of the high concentration of the chromophore (NAD) used in the Emit® st™ reagent, the reaction mixture was diluted 1/10 to measure the absorbance at 90 seconds after the initiation of the reaction with a Beckman spectrophotometer Model DU-64. To stop the reaction while the absorbance was being determined, 0.1 N of NaOH solution was used as diluent. Absorbance was measured every 90 seconds for 15 minutes.

RESULTS

Reaction Kinetics of the Emit® st™ Urine Amphetamine Assay with UV Spectrophotometer

The reaction kinetics of the Emit® st™ Urine Amphetamine Assay were determined with regular (3ml) or modified (5ml) protocols. The reaction mixture was diluted 1/10 and then determined with UV spectrophotometer (see Materials and Methods). The results of the regular protocol are presented in Figure 1. The reaction kinetics are almost linear at least for 15 minutes after the start of the reaction, reaching

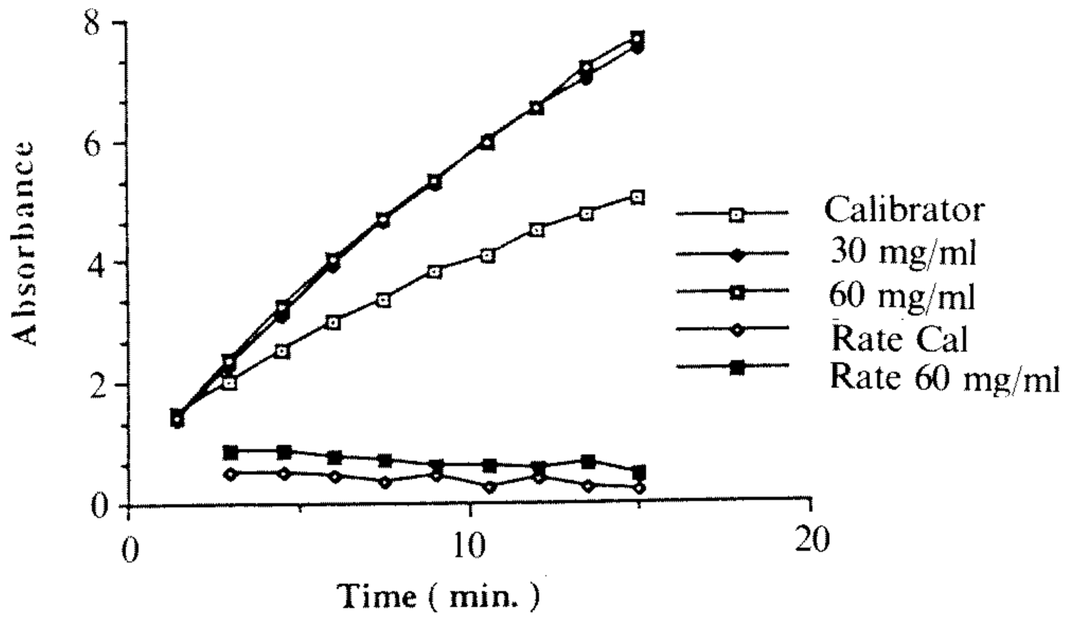

Figure 1 Reaction kinetics of Emit® st™ Amphetamine Assay with 3 ml reaction volume.

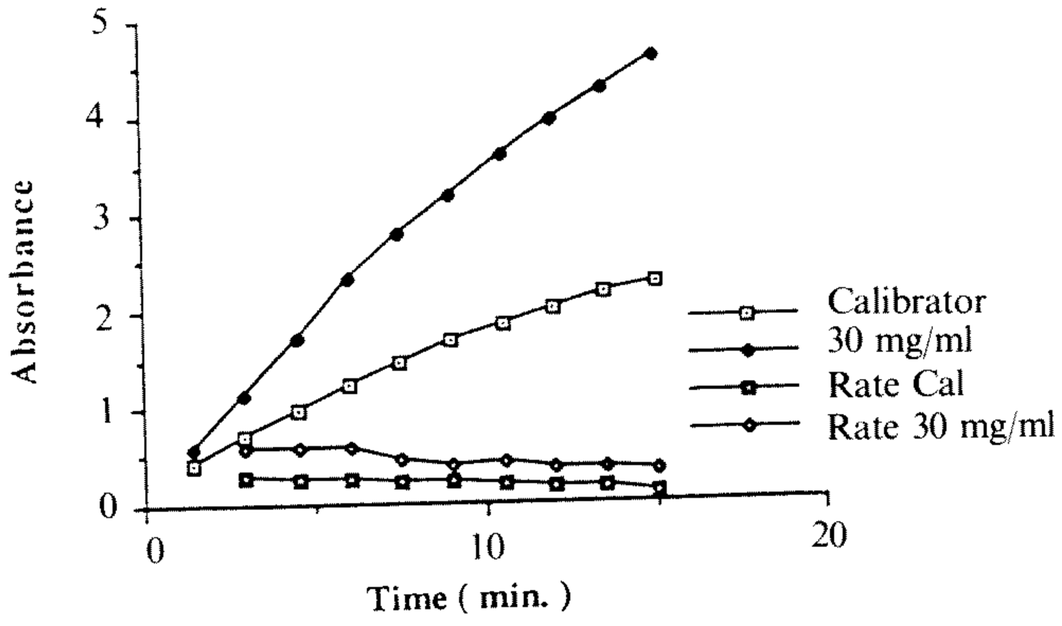

Figure 2 Reaction kinetics of Emit® st™ Amphetamine Assay with 5 ml reaction volume.

7 absorbance unit after 15 minutes. The kinetic curves of methamphetamine at 30 mg/ml and 60 mg/ml are superimposable. This indicated that at this extremely high concentration, maximum inhibition of antibody (and thus maximum enzyme activity) was achieved. The rates of the reaction calculated as the difference of absorbance between two consecutive measurements indicated that the rate of the positive control is always greater than the rate of the calibrator for the entire period monitored (15 minutes).

Because of the rapid increase of absorbance , a modified procedure with an additional 2 ml of distilled water was used to dilute and slow down the reaction. The results in Figure 2 indicated that the reaction rates were lower, and the rates of the positive control were always greater than the rates of the calibrator for a period of at least 15 minutes.

Repeated Determination of Emit® st™ Urine Amphetamine Assay with Emit® st™ Drug Detection System

To find out whether the timing of the reaction rate determinations affected the results, experiments were set up to measure the rates repeatedly with an Emit® st™ Drug Detection System. The calibrator, negative sample and samples of very high methamphetamine concentration (30 mg/ml) were assayed with regular or modified protocols, and the same pair of vials were measured repeatedly. The results are summarized in Table 1. The same pairs of calibrator and sample were measured 90 seconds after the initiation of reactions and repeated four times at 90-second intervals. The reaction rates of both vials decreased with the increase of time while the negative sample remained negative, and the positive sample remained positive when compared to the calibrator. With the 3 ml protocol, the reaction reached a point where the instrument could no longer accurately determine the rates of the reaction at the fourth measurements while the 5 ml protocol was still doing quite well. This indicated that more than one sample can be grouped together and assayed with one calibrator.

The results in Table 2 summarize 27 samples (18 positives and 9 negatives) which were assayed with the regular protocol and measured repeatedly with a Syva Drug Detection System for three consecutive times after the initiation of the reactions. The rates of the calibrators decreased with time to 92.2% and 81.2 % of the first measurement for the second and third measurements, respectively. However, the ratio of the reaction rates between the samples

and the calibrators remained constant.

Results of the Modified Protocol

The protocol was modified by a) the addition of an extra 2 ml of distilled water to the reaction and by b) assay of three samples with one calibrator. The rates of the samples were measured against the same calibrator one after another. The reaults from 64 samples are summarized in Table 3. The rates of the same 64 samples obtained with the regular protocol (3 ml) are included for comparison. The 56 positive samples were grouped according to the order each sample presented in the assay. The eight negative samples were grouped together irrespective of the order in the assay. The results of the samples do not change from positive to negative or vice versa. For positive samples, the ratio of the rates between the samples and the calibrator for the modified protocol were greater than those of the regular protocol. For the negative samples the ratio of the rates between the samples and calibrator were similar between the two protocols.

DISCUSSION

The purpose of this study was to modify the Emit® st™ Urine Amphetamine Assay currently in use in Taiwan to achieve a more efficient use of the expensive reagents. As in all enzyme reactions, the reaction rate of the Emit® st™ Assay levels off with the increase of time because of substrate depletion (Figs. 1,2; Table 1);thus the reaction rates measured earlier were greater than the rates measured later in the reaction. Since the Emit® st™ Amphetamine Assay is a competitive inhibition assay, the greater the concentration of the analyte, the higher the reaction rate. It is possible that at very high analyte concentrations, substrates may be depleted quickly. If this happens, the rates of samples with high concentration might be lower than the rate of the calibrator with lower concentration, if rates were both measured later in the reaction and led to wrong diagnosis. Present results indicated that this was not the case (Figures.1,2; Table 1). The rate of the reaction at methamphetamine concentration of 30 mg/ml is the same as those at 60 mg/ml (60 mg/ml is 200, 000 times the concentration of the calibrator, which contains 300 ng/ml of d,l-amphetamine), indicating that maximum inhibition and maximum enzyme activity was reached. The rate of methamphetamine at 30 mg/ml measured at 15 min. is still greater than the rate of the calibrator measured at the same time.

The absorbance of the assay increased very quickly, reaching 7 absorbance units in 15 minu-

Table 1. The Reaction Rates of Repeated Measurement of Emit® st™ Urine Amphetamine Assay with Emit® st™Drug Detection System

Order of Determination	Absorbance Change (mA/1.5 min.)					
	3 ml protocol			5ml protocol		
	Cal	Neg	Pos	Cal	Neg	Pos
1st	500	465	807	237	217	386
2nd	459	422	788	219	200	396
3rd	407	381	559	203	186	380
4th	396	347	+0+	186	173	341
5th	n.d.	n.d.	n.d.	175	n.d.	295

Abbreviations:

Cal:Calibrator, Neg:Negative control, Pos:Positive control

n.d.:Not done.

Table 2. Repeated Measurement of the Emit® st™ Urine Amphetamine Assay with the Regular Protocol and Unknown Samples

Order of Determination	Mean Absorbance Change (mA/1.5 min.)*		
	Calibrator	Positive	Negative
1st	582.1	686.9(1.18)**	494.8(0.85)**
2nd	536.8	648.1(1.21)**	451.4(0.84)**
3rd	422.7	553.5(1.17)**	402.7(0.85)**

*Mean reaction rates are the average of reaction rates of different samples individually tested (18 positives and 9 negatives).

**Numbers in parentheses are the ratio of the rates of samples to the rate of calibrator.

Table 3. Emit® st™ Urine Amphetamine Assays with Modified Protocol

Sample Order	Calibrator**	Rate Sample/Rate Calibrator	
		5 ml	3 ml
Positive			
1st(19)*	219.1	1.41	1.29
2nd(18)*	207.3	1.45	1.29
3rd(19)*	193.7	1.39	1.28
Negative(8)*		0.96	0.94

*Numbers in parentheses indicated the number of samples tested in the group.

**Numbers presented as the mean absorbance change in (mA/1.5 min.).

tes. The maximum absorbance was 12 units at the completion of the reaction (data not shown). The Syva Drug Detection System was unable to measure absorbance greater than four absorbance units. This point was reached at around the fourth determination (6 to 7.5 minutes after the initiation of the reaction) with the regular protocol (Table 1).With the modified (5ml) protocol, there was no problem at least up to the fifth determination (7.5 to 9 min.). Correct diagnosis was obtained with negative and positive controls at the fourth and fifth determinations for the 3 ml and 5 ml protocols, respectively.

To limit the delay between the start of the calibrator and the samples, we chose a protocol of one calibrator and three samples; the delay between the reaction initiation was less than 30 seconds between the calibrator and the third sample. As long as the number of samples to be assayed together was limited, with one calibrator , to fewer than three, both protocols are satisfactory.

Normally, six vials of reagents were needed to assay three samples. With the current modification (one calibrator and three samples), four vials are required to assay three samples. A saving of two vials (one-third of the reagent) is easily achieved with both 3 ml and 5 ml protocols. In theory, more samples could be run with a single calibrator to achieve even more savings with the modified (5 ml) protocol. Whether this approach is practical remains to be tested.

It is important to compare (a) the "sensitivity" (change of response per unit change of analyte concentration) of the original and modified protocols and (b) the effect of sample position on the "sensitivity" of the modified protocol. These parameters need to be evaluated before the modified protocol is adapted for routine use.

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Syva 安非他命篩檢測試的改良

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摘　要

目前在台灣有許多衛生局的檢驗單位使用Syva st試劑作尿液中安非他命的篩檢，一次只能作一個檢體，而且每作一個檢體時需要同時作一個標準液，每篩檢一個檢體的試劑費用約需台幣260元。我們經過分析改良後發現在加入檢體(或標準液)和水後的15分鐘內，吸光值的增加幾乎與時間的增加成直線的關係。使用目前的作業方式，在第一次比色得到結果後，同一組標準液和檢體如繼續比色，到第三次時雖然反應速度有顯著的減少，但仍可得到正確的答案。第四次比色時則因吸光值已超過儀器測試的上限，無法得正確的反應速度數值，但結果仍是正確的，如果在反應中多加2c.c.的水，則會使反應速度減慢，在第五次比色時仍可得到正確的結果。由此推論，我們可以在每次測試時使用一個標準液和三個檢體，檢體輪流和同一個標準液比色而得到結果，利用這種改良的方法(5c.c.)，我們發現至少可以把三個檢體和一個標準液一起測試，而節省三分之一的試劑和費用。在64個檢體的測試中，改良方法和原來的方法所得到的結果，彼此完全符合。