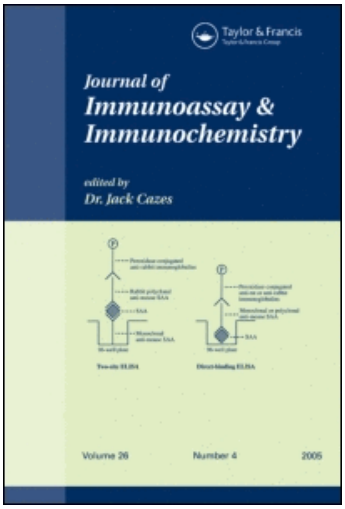

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COST EFFECTIVE EMIT ASSAYS, FOR DRUGS OF ABUSE IN URINE, USING THE EPPENDORF EPOS ANALYSER

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ABSTRACT

Laboratories with an increasing work load of drug abuse testing require high turnover techniques at low cost. Syva's EMIT system is suitable for modification onto modern automated instrumentation and, with reagent dilution, costs can be significantly reduced. We describe the modification of these assays onto the Eppendorf EPOS analyser which can process 300 samples an hour at a cost of 13p per test.

INTRODUCTION

Screening for drugs of abuse is a growth area in laboratory analysis. Because of the large numbers of samples being processed, initial screening techniques must be rapid and cost effective. Although confirmation of positive samples is required, immunoassays have become the method of choice for screening, with enzymoimmunoassays (Syva's EMIT [1]) and fluoroimmunoassays

(Abbott TDx [2]) widely used. However, there has been a recent drive towards greater cost effectiveness, as screening by these methods, though rapid, can be expensive. Because of the lack of modifiable fluorescent instrumentation little progress has been made with fluoroimmunoassays. The most widely used fluoroimmunoassay (Abbott TDx) is limited by small batch analysis (20 samples) and is not amenable to user modification. Recently several groups have successfully developed fully automated assays using EMIT reagents and, by use of reagent dilution, have greatly reduced costs [3,4,5]. Dilution of EMIT reagents is limited by substrate concentration present in the original kit [5,6] and dilution of EMIT reagents has been achieved by using buffer containing additional substrate. We describe the cost effective adaptation of the Syva system onto the Eppendorf EPOS analyser.

MATERIALS AND METHODS

Reagents

EMIT reagent kits for opiates, cocaine and benzodiazepines, together with relevant calibrators were from Syva, Maidenhead, Berks., U.K.; D-glucose-6-phosphate (G6P), and nicotinamide adenine dinucleotide (NAD^+) were from Sigma, Poole, Dorset, U.K. Lyphochek urine toxicology control was from Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.

G6P-NAD^+ reagent

D-Glucose-6-phosphate 5.16g and NAD^+ 12.14g were dissolved in 50 mL distilled water to give molar concentrations of 366 mmol/L of each constituent, and stored at -20° C in 0.5 mL aliquots.

Assay buffer

All working reagents were prepared in Tris HCl buffer (55 mmol/L, pH 8.0) containing NaCl (130 mmol/L), Triton X-100 (0.1 mL/L) and sodium azide (0.5 g/L).

Equipment

The Eppendorf EPOS instrument (BDH, Poole, Dorset, U.K.) is a programmable discrete analyser capable of kinetic and end-point analyses at high throughput. Biological fluids are sampled from sealed microcentrifuge tubes placed in a flexible chain, and are diluted with analytical reagents in laundered quartz cuvettes. Optics are based on a mercury lamp and interference filters. We used 334 nm being the nearest to the 340 nm recommended by Syva. Results can be calculated from a factor, a single standard, or a series of standards, linearly or by Spline interpolation curve fitting.

METHODS

Manufacturers procedure

Eppendorf publish a method for EMIT therapeutic drugs on EPOS [7] and this has been used to run EMIT d.a.u assays. EMIT reagent A (antibody/substrate) is diluted 17 fold with working buffer and used as reagent 1. Reagent B (enzyme) is used neat as reagent 2. With the reagent volumes used on the instrument this is effectively a three fold increase in the number of tests available from a kit. The EMIT calibrators were placed in the standard wells on top of the container for reagent 1, and a standard curve run and stored in memory. In working practice once a standard curve has been established, only a zero standard need be analysed at the start of each run of samples. This is done by using a drug free standard (negative calibrator) in a special section of the chain just prior to the samples which, effectively, adjusts the zero point of the standard curve without changing the absorbance difference between standards. In everyday use positive, negative and cut-off controls are assayed with the tests. Results are assigned as positive for concentrations above the cut-off, and negative for those below.

Modified EPOS procedure

EMIT reagent A was diluted 40-fold with assay buffer, to which was added 20 µL/mL G6P/NAD^+ (366 mmol/L), and this mixture

Table 1.

EPOS instrument settings for EMIT assays

	Eppendorf method	Proposed method
1 Name	drug	drug
2 Unit	µg/mL	µg/mL
3 Method	-----	-----
4 Sample Cannula	1	1
5 Endpoint	no	yes
6 Kinetic	yes	no
7 Meas. sec	7	n/a
8 Incub. sec	12	120
9 Preincub. sec	156	36
10 Cycle sec	12	12
15 Sample-blank	n/a	yes
16 Reagent-blank	no	no
19 Constant	0	0
20 Cal. curve	yes	yes
22 Std 1-conc	0.00	0.00
23 Std 2-conc	0.30	0.30
24 Std 3-conc	1.00	1.00
25 Std 4-conc	0	0
26 Std 5-conc	0	0
27 Std 6-conc	0	0
30 £ of std meas	2	2
31 £ of sample meas	1	1
32 Sample uL	3	5
33 Reagent 1 uL	290	240
34 Reagent 2 uL	17	48
35 Limit	1000	1000
36 Auto-Repeat	no	no
38 Control 1	no	no
40 Control 2	no	no
42 Control 3	no	no
44 Temperature ^{o}C	30	30
45 Wavelength nm	334	334
46 Adjust A	0.0	0.0
54 Samples/h	300	300
55 Timesharing	yes	yes

was used as reagent 1 on the analyser. Reagent B was diluted eightfold with buffer and used as reagent 2 on the analyser. This procedure enabled 1000 assays from each 100 assay Syva kit.

A comparison of the instrument settings used in the two methods is given in Table 1.

Specimens

One hundred urine samples were obtained from the Department of Chemical Pathology, Hackney Hospital. All were from patients attending a drug dependency clinic and had previously been tested for opiates by thin-layer chromatography (TLC) [8] and for benzodiazepines and cocaine by TDx.

RESULTS

Effect of additional G6P/NAD^+ on enzyme activity

Dilution of Emit reagents is limited by substrate concentration in the original kit [5,6] and a ten fold dilution of reagent A reduced enzyme activity to an unacceptable level. Recovery of enzyme activity was achieved by adding additional substrate to diluted reagent A (Figure 1) [5]. In the proposed method modification we use an additional 20 µL/mL of a 366 mmol/L solution of G6P/NAD^+, giving a final concentration of 7.3 mmol/L. The results compare favourably with other reports [5] with an increase in absorbance change of at least two fold by adding more than 6 mmol/L G6P/NAD^+.

Comparison of standard curves

Standard curves were run using both procedures (Figure 2).

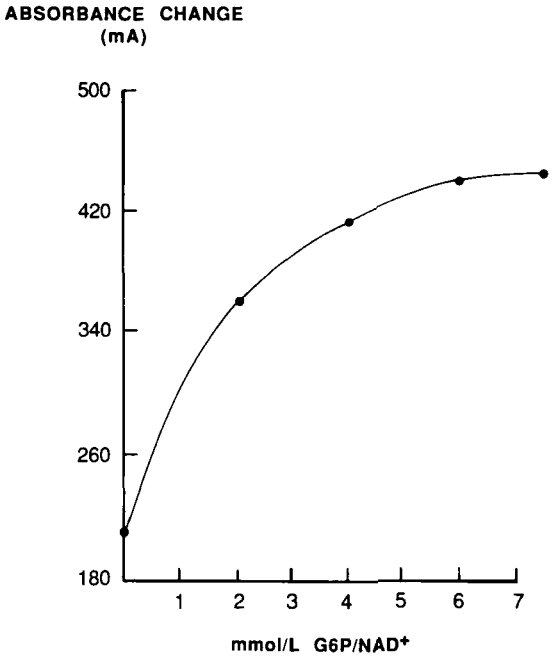

Figure 1

Increase in sensitivity using additional G6P/NAD^+ in diluted reagents.

Measurements are based on the increase in absorbance of an EMIT calibrator B used in a cocaine metabolite assay. Similar changes are seen with all the kits available. Zero is equivalent to the EMIT kit diluted with no addition of extra substrate.

Assay Validation

Twenty replicates of a commercial urine toxicology control, a five fold dilution of the toxicology urine and a known drug free urine were assayed by all three modified kits. The control urine contained 2 mg/L oxazepam, 3 mg/L benzoylecgonine and morphine,

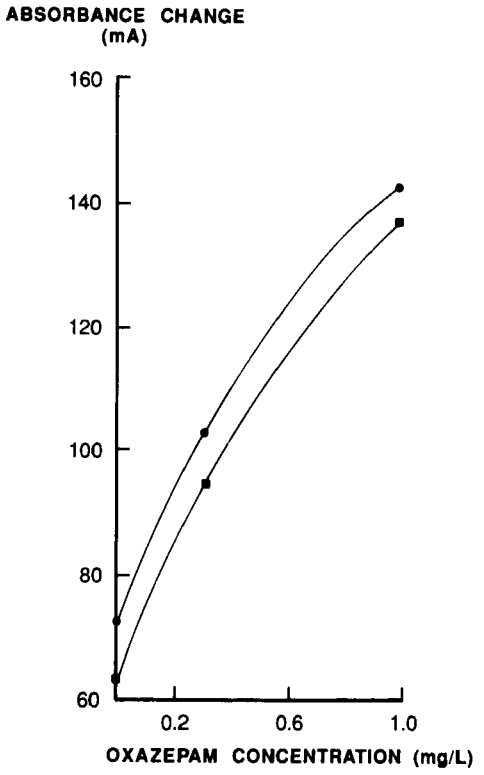

Figure 2

Standard curves using the Eppendorf method compared to the proposed method.

proposed method

Eppendorf method

and 1 mg/L codeine. Within batch precisions using the urine diluted five fold were 1.5% for benzodiazepines, 3.8% for benzoylecgonine, and 1.1% for opiates. Using an undiluted control they were 1.9% for benzodiazepines, 3.5% for benzoylecgonine, and 2.2% for opiates. Between batch precision was assessed using the Low and Medium calibrators on successive runs over the course of several weeks without change in calibration curve when using the zero readjustment feature. Coefficients of variation using the Low calibrators (0.3 mg/L) were 3.3% for benzodiazepines, 4.7% for benzoylecgonine, and 3.1% for opiates. Those for the Medium calibrators (1.0 mg/L) were 3.8% for benzodiazepines, 4.3% for benzoylecgonine, and 3.5% for opiates.

Minimum detection limits [9] were 20 µg/L for benzodiazepines and cocaine metabolite, and 30 µg/L for opiates.

Correlation with alternative methods

One hundred samples from a drug dependency clinic were assayed for opiates, benzoylecgonine, and benzodiazepines. These samples had been previously analysed by our existing techniques. Complete agreement was achieved between techniques for benzodiazepines and benzoylecgonine while the EMIT opiate assay detected 38 positives whereas TLC found only 26. The twelve samples negative by TLC were found to be positive after hydrolysis.

Table 2

Comparitive costs of EMIT d.a.u. reagents

Method	Tests/kit	Reagent Cost/test (£)	Consumable Cost/test (£)	Total Cost/test (£)
Syva manual	100	1.20	0.02	1.22
Eppendorf	334	0.36	0.01	0.37
Cobas Bio	334	0.36	0.05	0.41
Proposed EPOS	1000	0.12	0.01	0.13
Sung & Neeley [5]	2470	0.05	0.05	0.10

Consumable costs include sample cup and reaction cuvette where appropriate.

Cost effectiveness of diluted EMIT kit

We have compared the cost of the proposed modification with others recently published (Table 2). The costs include the price of the in-house supplementary substrate reagent. The reduction in price from the original manual technique is almost ten fold which is comparable to other published modifications.

DISCUSSION

Screening procedures for drugs of abuse need to eliminate negative samples quickly, accurately, and cheaply. Three EMIT assays were used in this evaluation and adapting them onto the EPOS analyser produced rapid, cost effective, assays. The

instrument has a coefficient of variation under 1% (absorbance units) in routine use, and can process 300 samples an hour. Once a standard curve has been established, we found the absorbance values to be constant for a batch of reagents when using the zero readjustment feature. Re-standardisation is only necessary when reagent batch numbers alter. Working reagent stability was at least two weeks which is more than adequate for large batch processing. Similar working reagent stability for at least two months has been reported by others [5], although they recalibrated the assay for each sample run. The instrument parameters for the proposed method (Table 1) are used in our laboratory for all the current range of EMIT d.a.u assays.

Within, and between, batch precision is comparable to published methods for other instruments where within batch precision is around 3 %, and between batch is around 5% [10,11]. We assessed the effect of further dilution of EMIT reagents on EPOS, and found that precision worsens by a factor of at least two when 1500 tests are obtained and deteriorates still further if 2000 tests are obtained. The absorbance changes also become unacceptably low when the kit is diluted to this degree, even with the extra substrate reagents present. In day-to day usage it was found to be a very reproducible assay even when used by junior laboratory staff. Good correlation with existing methods was achieved and we were able to reduce the cost to 13 pence a test which is a considerable cost saving, comparable to other suggested modifications.

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