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Short Communication

Evaluation of enzymic assay for paracetamol: clinical and forensic experiences

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Abstract: The performance of the enzymic paracetamol assay (Cambridge Life Science, UK) was evaluated for use in clinical and forensic analyses and compared with gas–liquid chromatography and EMIT (Syva) systems. It was found to be precise, accurate and rapid for the analysis of paracetamol in serum, with a possible application for the quantitative screening of post mortem blood.

Keywords: *Enzymic paracetamol assay; EMIT–GLC comparison; clinical applications; forensic applications.*

Introduction

Paracetamol is taken in 25% of accidental and self-administered poisoning cases admitted to the casualty department in the hospital where the authors' laboratory is sited. Toxicological investigations were carried out on post mortem (PM) cases from the mortuaries in north and west London over the period of 1978–1982. They showed that paracetamol was present in approximately 20% of all cases in which drugs had been detected (S. C. Paterson, personal communication, 1983).

Paracetamol assays present heavy demands on staff time and a rapid quantitative screening technique is required. This is particularly so in the case of emergency toxicological screening where paracetamol levels in blood are important in assessing possible liver damage and any necessary antidote treatment [1, 2]. Forensic toxicology also requires that a drug is identified and measured by at least two different techniques.

Several methods are available — gas–liquid chromatography (GLC) [3], high-performance liquid chromatography (HPLC) [4], EMIT (Syva, P.O. Box 10058, Palo Alto, Ca.) and colourmetric techniques in their various forms (particularly that of Glyn and Kendal) [5]. The advantages and disadvantages of all these methods have been reviewed [3–7]. Two methods using EMIT and GLC have been compared with a recently marketed enzymic paracetamol assay (Cambridge Life Science, UK).

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This assay utilizes a bacterial aryl acylamide amidohydrolase converting paracetamol at room temperature to *p*-aminophenol, producing a blue coloured indophenol complex, which may be quantified spectrophotometrically. The enzymic assay was assessed for any advantages over established techniques for use with specimens taken from live patients and corpses.

Experimental

Apparatus

All spectrometric measurements relating to the enzymic method were made on a Unicam SPI800 ultra-violet spectrometer.

The EMIT paracetamol assay was carried out using a Gilford Stasar III Spectrometer in conjunction with a semi-automated Syva CP-5000 clinical processor system.

Gas chromatography was done on a Varian 3700 gas chromograph fitted with a model 8000 auto sampler and coupled to a Spectra-Physics SP4100 integrator. A 4ft × ⅛ inch i.d. glass column was packed with 3% OV-17 on Gas Chrom Q (100–120 mesh) and operated at oven temperature 165°C, injector temperature 240°C, detector temperature 260°C and with a nitrogen carrier gas flow rate of 30 ml/min. A thermionic specific detector (TSD) was used.

Samples were centrifuged at low speed using an IEC centra-7 centrifuge or at high speed using an Eppendorf J414 centrifuge.

All pipetting was carried out using Eppendorf, Socorex, Standard glass or Hamilton Micro Lab M pipetting systems.

Materials

Enzymic paracetamol assay kit (Cambridge Life Sciences UK); EMIT acetaminophen serum assay kit (Syva); supply of drug-free expired whole blood (Haematology, Charing Cross Hospital); serum samples from casualty admissions (Charing Cross Hospital) and freeze-dried paracetamol specimens for quality control (Dudley Road Hospital, Birmingham, UK).

The following reagents were used as Analar grade: diethyl ether (May & Baker), 10% trichloroacetic acid (TCA) (BDH), tetramethylammonium hydroxide (TMAH) (BDH, Poole, UK), methanol (May & Baker). Subtilisin Carlesberg (was obtained from Nova Industri A/S), 1 m hydrochloric acid (HCl) was obtained from Fisons, *n*-butryl *p*-aminophenol and all water used was glass-distilled.

Procedure

(i) *Assays on spiked specimens to measure precision.* Serum from normal individuals was spiked with paracetamol (50 and 200 μg/ml) and assayed by the three systems.

The protocol supplied with the kits was used for the enzymic and EMIT methods. For the GLC method, standards were made up to cover a range of 0–500 μg/ml using 1 ml normal serum and aqueous paracetamol solution (for samples, 1 ml serum and 1 ml water taken). To 2 ml test solution, 0.5 ml internal standard solution (200 μg/ml *n*-butyl *p*-aminophenol), 0.2 ml 1 M HCl and 6 ml diethyl ether were added. After shaking and centrifuging (1500 ***g***, 5 min) the organic layer was removed, evaporated to dryness and reconstituted using 0.5 ml of 1:80 TMAH solution (v/v) in methanol, injecting 1 μl onto the GLC.

(ii) *Assays on sera from live patients.* Assays using the three analytical methods were carried out on 40 specimens 75% of which were obtained from patients admitted to the casualty department; the remainder were freeze-dried quality controls.

(iii) *Assays on post mortem samples.* Three methods of sample preparation were used on post mortem blood known to contain paracetamol and whole post mortem blood spiked with different amounts of paracetamol was used untreated and with three types of pretreatment as follows.

(a) Protein precipitation with TCA. Equal amounts (0.1 ml) of sample and 10% TCA were used and centrifuged (9000 ***g*** for 1 min); 0.05 ml of the supernatant was then assayed as described previously.

(b) Treatment with subtilisin Carlesberg before protein precipitation. The sample (0.1 ml) was incubated with 0.1 ml subtilisin Carlesberg (1 mg/ml in H_2O pH 7.0) for 30 min at 60°C; 0.1 ml 10% TCA was added and after mixing and centrifuging (9000 ***g*** for 1 min) 0.05 ml of supernatant was assayed as previously.

(c) Extraction of paracetamol for enzymic method. To 1 ml sample and 1 ml water, 0.2 ml 1 M HCl and 6 ml diethyl ether were added. After shaking (10 min) and centrifuging (5 min at 1500 ***g***) the organic layer was removed and evaporated to dryness. Reconstitution was effected using 0.2 ml methanol and 0.5 ml water; 0.05 ml of this solution was assayed using the enzymic method.

The standard paracetamol solution was made up in blood at a concentration of 300 μg/ml.

For each method of sample preparation GLC was used as a reference method. The system used was the same as that described earlier, except that the standards (concentration range 0–500 μg/ml) were made up in blood.

(iv) *Possible interference with vitamin C.* Aqueous solutions of paracetamol at concentrations of 50, 200 and 400 μg/ml were spiked to give different concentrations of vitamin C ranging from 0 to 2000 μg/ml. Each sample was then assayed using the enzymic method.

Results

(i) *Precision*

The mean results (±SD) obtained on the two samples, 50 μg/ml and 200 μg/ml using the enzymic, EMIT and GLC methods respectively were 50.2 (±2.8), 47.4 (±2.8) and 53.2 (±1.6) for the 50 μg/ml sample and 197 (±5.2), 195 (±15.8) and 184 (±4.5) for the 200 μg/ml sample.

(ii) *Samples from live patients*

Figure 1 compares the results of the three types of assay on 40 serum samples. The number of samples used for each type of assay was limited by the sensitivity cut-off point. Negative values found using two of the types of assay were not used in the comparison. The limit of detection for the enzymic assay was 10 μg/ml.

(iii) *Post mortem samples*

Table 1 gives the mean value obtained for paracetamol in post mortem blood and spiked blood using the enzymic method and GLC. The ratio enzymic/GLC (reference) results is given as a percentage.

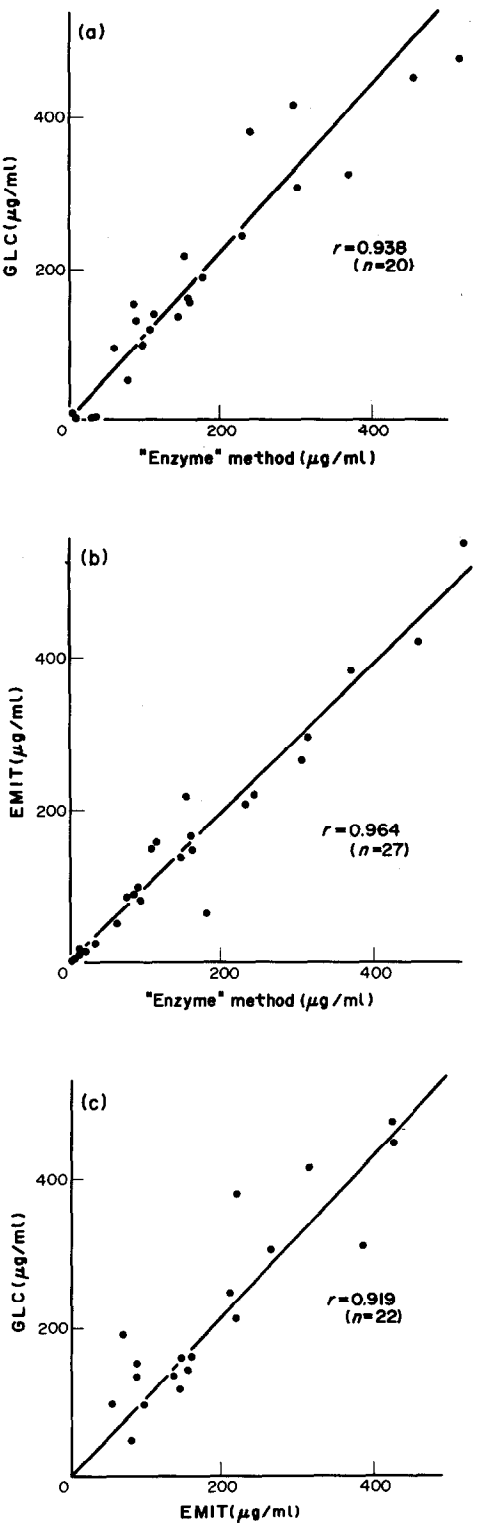

Figure 1
Correlations between (a) the enzyme method and GLC, (b) the enzyme method and EMIT, (c) EMIT and GLC for paracetamol.

Table 1

Sample preparation	Method	Spiked blood (μg/ml) 50	100	200	Post mortem blood (μg/ml)			Mean % enzymic/GLC*
None	Enzymic	41	66	144	93	123	168	
	GLC	37	91	201	161	212	183	
	Enz/GLC (%)	111	73	72	58	58	92	77
10% TCA	Enzymic	19		136	147			
	GLC	40		201	161			
	Enz/GLC (%)	48		68	91			69
Subtilisin + 10% TCA	Enzymic	43	86	144	185	151	163	
	GLC	37	91	201	212	183	161	
	Enz/GLC (%)	116	95	72	87	82	101	92
Ether extraction	Enzymic	41	97	203	156	149	149	
	GLC	40	89	186	163	149	242	
	Enz/GLC (%)	102	109	109	96	100	62	96

(iv) *Possible interference with vitamin C*

At each level of paracetamol (50, 200 and 400 μg/ml) the concentration of vitamin C had no effect on the assayed paracetamol level.

Discussion

The experiment using blood from live patients compared the performance of the enzymic with EMIT and GLC methods on 40 serum samples. Since most of the specimens were from patients admitted to casualty, a range of paracetamol levels was covered, sometimes in combination with other drugs. The results show that the enzymic method compared favourably in terms of accuracy and precision with EMIT and GLC. This has also been shown for a spectrometric assay [8].

One possible source of interference with the assay was thought to be Vitamin C. Swale [9] reported that high levels of this compound in urine produced false negative results with the *o*-cresol reaction. The trial showed that over a concentration range of one hundred times its physiological level, in aqueous solution there was no evidence of vitamin C interference using the enzymic method.

Analysis time for assaying a sample using the enzymic method was 15–25 min compared with 15–45 min for EMIT (the lower figure depends upon the use of a clinical processor unit which can hold a calibration curve for a number of days) and at least 1 hour for a non-dedicated GLC system. The method therefore has an important advantage in this respect for emergency work.

These levels are interpreted on the basis of Prescott [1], who showed that the severity of liver necrosis was related to the level of serum paracetamol at a known time from the ingestion of the tablets. Antidote treatment is ineffective unless administered within 10–12 h of paracetamol ingestion. With this rapid enzymic method, the patient can receive treatment after a shorter delay than would normally have been experienced, thus reducing the possibility of liver damage. The sensitivity of the assay (down to 10 μg/ml) enables the detection of drug ingestion by the occasional patient who arrives in casualty more than 12 h after taking a potentially fatal overdose, but before significant biochemical changes can be detected. The patient can then be retained in hospital for observation.

Running costs of the enzymic method were approximately half of those for EMIT. However, this was dependent on the number of samples assayed, how often they arrived and whether it was possible to avoid recalibrating each time an assay was carried out on the EMIT system.

The enzymic method was shown to work directly on PM blood, but the recoveries were low. Protein precipitation with 10% TCA failed to improve recoveries, perhaps as paracetamol was trapped in precipitated protein. However, direct solvent extraction or digestion with subtilisin Carlesberg before TCA precipitation gave recoveries comparable to those using the reference GLC method. This may be due to a better release of protein-bound paracetamol and less spectrometric interference since both methods produce supernatants with low optical densities.

Conclusion

The enzymic method for paracetamol assay is sufficiently precise, accurate and rapid for clinical use. It requires no special equipment and is easy to perform. It is not affected by Vitamin C and is suitable for use on post mortem blood.

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