TITLE
Reversing microcrystalline tests - An analytical approach to recycling of microcrystals from drugs of abuse

AUTHORS
Leonie E. Elie[1] *
Dr. Mark G. Baron[1]
Dr. Ruth S. Croxton[1]
Mathieu P. Elie[1]

[1] University of Lincoln, School of Natural and Applied Sciences, Lincoln, LN6 7TS, UK; phone: 0044 1522 886855; email: lelie@lincoln.ac.uk
* Corresponding author

ABSTRACT
A combined analysis of microcrystalline tests followed by LC-MS or GC-MS analysis is described. Microcrystalline tests are shown to be non-destructive as addition products formed were easily dissociated after the application of an appropriate solvent. Subsequent analysis of the sample was done to quantify the recovery of the drug. Examples were performed using the date rape drug γ-hydroxybutyrate (GHB) and the synthetic opioid methadone.

INTRODUCTION
Microcrystalline tests are chemical tests where microscopic crystals are formed upon the mix of a specifically chosen reagent together with a suspected drug sample. They provide a simple answer as to whether the suspected drug is present or not and can be carried out at very little cost. No specialist analytical instrumentation is required to perform a successful test; only a microscope is needed in order to observe the result. Result interpretation is based on direct crystal comparison with genuine drug standards which are carried out alongside [1].

Microcrystalline tests, as well as colour tests, are commonly used for presumptive testing of suspected drug samples [2]. Only a few grains of suspected drug are required for a positive result. However, microcrystalline tests can be more than just a simple screening technique. A recent publication suggests that microcrystalline tests can be used as a confirmation technique [3]. As microcrystals vary in appearance depending on the drug and the reagent, characteristics like crystal habit, colour and shape are specific enough to give confirmation of a result.

Microcrystals formed between drugs of abuse and their corresponding reagents have been reported to be the result of addition reactions where drug and reagent form a complex [3]. The bond between drug and reagent molecules could be broken easily by adding solvent onto the crystals, dissolving the crystals and releasing the drug molecules back into solution. Solvents in which the analyte has optimum solubility are the most appropriate to chose.
Following this theory, microcrystalline tests would be reversible and non-destructive in the sense that the analyte molecule can still be detected by other techniques even though the sample may be contaminated with reagents. The recycling of samples could be of use where seized samples have been insufficient to carry out confirmation tests.

This study investigated a procedure to re-use microcrystalline tests for follow-up analysis by GC-MS or LC-MS. To demonstrate the effectiveness of the procedure two drugs of abuse, methadone and γ-hydroxybutyric acid (GHB), were chosen. Quantification was done using GC-MS and LC-MS to investigate the drug recovery.

MATERIALS AND METHODS
Chemicals and materials
γ-Hydroxybutyric acid sodium salt (99%) and methadone hydrochloride (> 98%) were purchased from Sigma-Aldrich (Gillingham, UK). GHB-d_6 and methadone-d_9 were purchased as ampoules (1 g/L in methanol) from Cerilliant (Round Rock, Texas, USA). Silver nitrate (99.9999%) was purchased from Sigma-Aldrich, lanthanum nitrate (> 99%) from Fluka (Gillingham, UK) and mercuric chloride from BDH chemicals (Poole, UK). Methanol (HPLC-grade) and formic acid (analytical reagent grade) were from Fisher Scientific (Loughborough,

UK). All microcrystalline tests were carried out on standard microscope glass slides purchased from Fisher Scientific.

Microcrystalline test

The reagent for GHB is an aqueous solution of silver nitrate and lanthanum nitrate (10 g/L each). Methadone was developed with an aqueous solution of mercuric chloride (10 g/L). Microcrystalline tests were set up by mixing 10 µL of drug (10 g/L in water) with 10 µL of the corresponding reagent on a glass slide. Progress of the microcrystal development was observed with a Meji ML 5000 microscope (Axbridge, UK) at 100 x magnification. The tests were left to fully dry at ambient temperature (drying time 15 minutes to 1 hour).

Sample recovery and preparation for instrumental analysis

The fully developed test slides were clamped onto a stand at an angle of approximately 10° to the horizontal for easy collection of the liquid sample. The solvent for dissolving the microcrystals was deionised water for both methadone and GHB. A 1.4 mL screw-cap vial was placed underneath one corner of the glass slide in order to catch the solvent running off the slide. In aliquots of 200 µL deionised water was smoothly run down the slide to dissolve all solids. Dissolving was sometimes aided by a gentle scratching movement done with a plastic pipette tip. A total volume of 800–1000 µL of deionised water was applied. Visual inspection of the rinsed slides with a microscope at 100 x magnification showed no crystal or other solid residues.

The collected sample solution was blown down to dryness under a stream of nitrogen at 40°C with a drying time of 60 to 90 minutes and reconstituted in water for GHB and methanol for methadone. Final concentrations assuming full recovery were 100 mg/L for GHB and 50 mg/L for methadone, respectively. The deuterated analogues GHB-d_6 and methadone-d_9 were added directly before analysis of the recovered microcrystalline test samples resulting in final concentrations of 100 mg/L for GHB-d_6 and 50 mg/L for methadone-d_9.

LC-MS conditions

GHB calibration standards were prepared in an aqueous solution ranging from 0 to 200 mg/L. GHB-d_6 was added as an internal standard just before injection resulting in a concentration of 100 mg/L.

Liquid chromatography was performed on an Agilent Technologies LC-MS system using a 1200 Series binary pump and an 1100 Series autosampler; it was operated by ChemStation software from Agilent Technologies (2005). Separation was achieved on an Agilent Technologies C18 column (Zorbax ODS 250 mm x 4.6 mm and 5 µm pore size) at ambient temperature. The column was eluted isocratically with 0.05% formic acid: methanol (90:10) at 0.5 mL/min using an adapted version of a previously reported method [4]. The injection volume was 1 µL.

Mass spectrometry was performed on an Agilent Technologies 6310 Ion Trap operated by 6300 Series TrapControl software from Bruker Daltonik (2008) in smart mode. Ionisation was achieved using Electrospray in positive ionisation mode (ESI^+). The ESI nebulizer was operated at 35.0 psi, drying gas nitrogen was run at 12.0 L/min with a drying temperature of 350 °C. The scan range was set to 50-2200 m/z with a target mass at 105 m/z for [M+1]. The target mass was changed accordingly when looking for GHB adducts. The compound stability was set to 10% due to the fragility of the GHB molecule and the trap drive level was operated at 50%.

GC-MS conditions

Methadone calibration standards were prepared in methanol ranging from 0 to 100 mg/L. Methadone-d_9 was added as an internal standard just before injection resulting in a concentration of 50 mg/L.

Gas chromatography was performed using a Perkin Elmer Clarus 500 Gas Chromatograph equipped with an autosampler and a capillary injector operated at 250°C in split mode (10:1). Using an adapted version of a previously reported method [5] separation was achieved on an Agilent Technologies DB-1MS column (30 m x 0.25 mm and 0.25 µm film thickness) starting at 150°C and increasing at 12°C/min to a final temperature of 300°C giving a total run time of 12.5 min. The carrier gas was helium at 1 mL/min and the injection volume was 1 µL.

Mass spectrometry was performed using a Perkin Elmer Clarus 500 Mass Spectrometer operated with Perkin Elmer TurboMass (2008) software. The transfer line temperature was

held at 250°C. Ionisation was achieved using an Electron Impact (EI) source at 200°C with electron energy of 70 eV. The multiplier was set to 480 V. After 2 minutes solvent delay the methadone and methadone-d_9 peaks were observed in total ion count (TIC) mode. Quantification was achieved by selected ion monitoring using the ions 72 m/z and 78 m/z at a dwell time of 0.2 s and a 0.1 s inter channel delay for methadone and methadone-d_9, respectively.

RESULTS AND DISCUSSION

The chosen microcrystalline and instrumental methods were selected because samples could be analysed underivatised which minimised sample loss, and saved preparation time and the cost for derivatisation reagents.

Microcrystalline testing of the drug sample

The microcrystalline test for GHB with silver nitrate and lanthanum nitrate as reagent showed previously reported GHB right angle crystals which were slowly nucleating from the periphery of the liquid drop towards the centre [6] [Figure 1A]. When the drop was almost dry, reagent crystals formed a coating layer upon all developed crystals.

The test for methadone developed differently. Methadone with mercuric chloride as reagent formed characteristic rosettes of branching rods [7]. When reagent and drug were mixed an instantaneous formation of white particles occurred. After a few minutes the drug-reagent crystals started to grow consuming the particle cloud as they increased in size. Only reagent crystals could be seen at the drying solvent front as methadone crystals preferred nucleation within the drop [Figure 1B].

All crystal test samples were left to dry completely to ensure maximum formation of drug-reagent crystals and to stay consistent throughout all experiments. However, it is normally advised that tests are observed well before complete dryness is achieved to avoid false results due to reagent crystal formation in the later stages of the test.

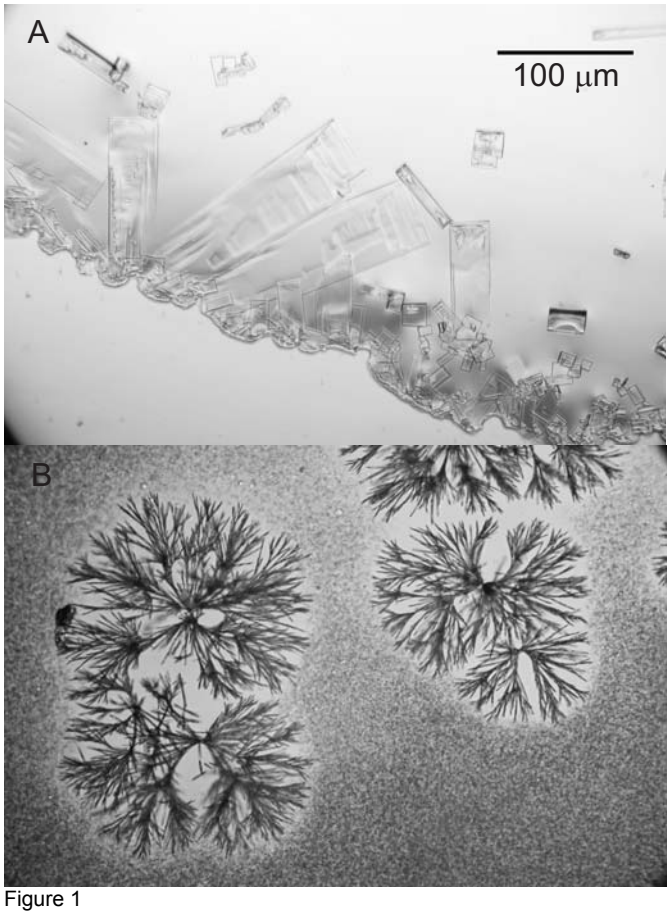

Figure 1
Microcrystals for A) GHB and silver-lanthanum nitrate reagent and B) methadone and mercuric chloride reagent

Recycling of the microcrystalline test material

In order to break up the bond formed between drug and reagent and therefore re-dissolving the drug molecules, solvent had to be applied. Deionised water was chosen because the majority of microcrystalline tests are carried out using aqueous solutions. Moreover, the viscosity of water as well as its surface tension make it the perfect solvent for this recycling procedure. The hydrophilic surface of microcrystals welcomed the applied water and disintegration of most crystals happened almost instantaneously. Water was applied in small aliquots of 200 µL to control the flow of solvent running over the edge of the slide [Figure 2]. Some crystal material did not dissolve in this step and was consequently washed off the slide in the stream of applied water. A total applied volume of 1000 µL deionised water was found to be sufficient to transfer the microcrystalline test sample analytically into a screw cap vial. Once transferred the final recycling step was to mix the solution with a vortex mixer for approximately 30 seconds inside the vial in order to dissolve all crystal material.

The recovered samples were blown dry under nitrogen at low temperature. It was necessary to achieve complete dryness in order to monitor the recovered quantities of drug correctly. Reconstitution solvents were chosen according to the instrumental analytical technique which was carried out subsequently on the samples.

No deuterated internal standards were used to monitor the microcrystalline test recovery. GHB-d_6 and methadone-d_9 are only available as solutions in methanol. Organic solvents like methanol have been found by the authors to interfere with the microcrystalline test.

A sample clean-up in order to remove the reagents prior to the instrumental analysis was not done and so reagents remained within the solutions throughout the analysis. Although interferences could not be detected, a simple precipitation step may be done to remove the metal cations by adding sodium iodide to form the insoluble complexes of silver iodide and mercury iodide which could then be easily filtered off.

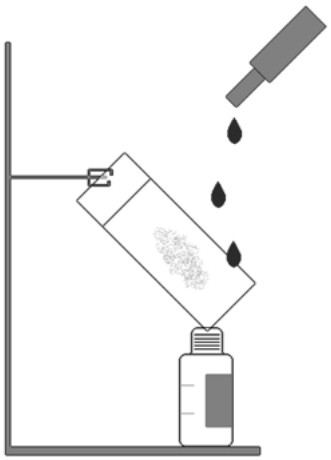

Figure 2
Schematic view of slide rinsing procedure for recycling of microcrystalline tests

Instrumental analysis of the recovered sample

Confirmation of the drug present was done via comparison of retention times and identification of characteristic ions in mass spectra obtained with standards. The retention times were 8.5 minutes for GHB and 7.9 minutes for methadone. Furthermore, mass spectrometry was used to quantify the drug recovery. Specific masses were monitored for the calibration and recovery study as pure drug standards were used throughout the experiments [Table 1]. The masses were chosen according to their high abundance in the standard mass spectra to give best sensitivity.

Table 1
Retention times and selected ions used for quantification of the microcrystalline test recovery

Compound	Retention time (min)	Ion (m/z)
GHB	8.5	87
GHB-d_6	8.3	93
Methadone	8.0	72
Methadone-d_9	7.9	78

LC-MS analysis of GHB

The mass spectra for GHB and its deuterated internal standard showed the molecular ion [M+1], one fragment due to the loss of water and common ESI^+ adducts [Table 2].

The base peak was used for quantification as it was the most abundant GHB ion in the spectrum. The molecular ion [M+1] was part of the background noise even at lowest ionisation settings in the ion trap presumably because the analyte desolvation before the trap was operated at 350°C. GHB was proven to be a thermally unstable molecule which dehydrates into a lactone [8]. The same behaviour was observed with the reconstituted microcrystalline samples which indicated that the bond formed between two GHB molecules and one silver ion (Ag^+) to form the right angle GHB crystal was broken [9]. No silver or lanthanum adduct could be detected at concentrations analysed.

Calibration linearity was achieved between 0 and 200 mg/L with a correlation coefficient of 0.993 [Figure 3A]. The sample recovery was calculated to be 103 % (n = 11 with CV% = 5 %).

Table 2
Typical ESI^+ ions for GHB and GHB-d_6

Ions	GHB (m/z)	GHB-d_6 (m/z)
Molecular ion [M+1]	105	111
Base peak	87	93
Na adduct	127	133
K adduct	143	149

GC-MS analysis of methadone

The mass spectra for methadone and its deuterated internal standard showed low abundance of the molecular ion but a high abundance for the m/z of 72 and m/z of 78 fragments, respectively [Table 3]. The ion of 165 m/z was common for both compounds and therefore not used for quantification.

Table 3
Typical EI ionisation ions for methadone and methadone-d_9

Ions	Methadone (m/z)	Methadone-d_9 (m/z)
Molecular ion	294	303
Base peak	72	78

The 72 m/z fragment was used for quantification even though on its own it is not sufficiently characteristic for methadone due to its small size. However, in conjunction with the beforehand performed microcrystalline test, the comparison of retention times and analysis of the TIC mass spectrum a definite identification was achieved.

Calibration linearity was achieved between 0 and 100 mg/L with a correlation coefficient of 0.999 [Figure 3B]. The sample recovery was calculated to be 105 % (n = 9 with CV% = 11%).

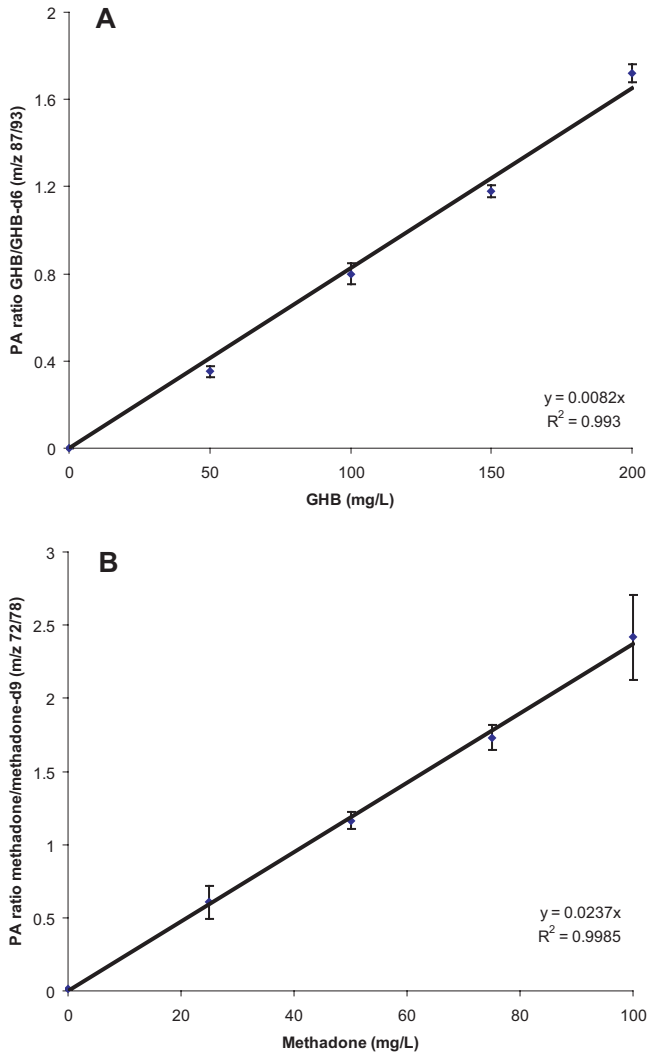

Figure 3
Calibration for A) GHB via LC-MS and B) methadone via GC-MS
Note: Peak area ratio (PA) of analyte divided by internal standard; *n* = 3; standard deviation as error bars

Result evaluation

The reagents themselves did not interfere with the GC or LC-MS results as only a few drug specific mass-to-charge ratios were evaluated in data treatment. Other detection techniques such as UV detection were not found suitable as the microcrystalline reagents interfered by absorbing in the UV.

It has been demonstrated in this study that the ionic bond formed during the process of crystallisation could be easily broken by applying the appropriate solvent onto the microcrystalline test sample. The proposed procedure used 0.1 mg of the solid drug sample and delivered presumptive testing, screening, confirmation and quantification. Depending on the sensitivity of the microcrystalline test applied and the purity of the tested sample, even a lower concentration of the drug sample can be used.

CONCLUSIONS

Methadone and γ-hydroxybutyrate were successfully recovered after performing microcrystalline tests. It was proven that microcrystalline tests are reversible and non-destructive. It was possible to use the microcrystalline test technique in conjunction with separation techniques to perform a successful qualitative and quantitative forensic drug analysis. Paired up both techniques can deliver a fast and reliable result using only a few micrograms of a suspected drug sample.

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