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# Multi-target Screening of Toxicological Compounds in Blood on A Fully-automated Platform Consisting of Sample Preparation Module CLAM and LC-MS/MS

Nat Tansrisawad[1], Udomsak Hoonwijit[1], Apinya Tubtimrattana[2], Boontariga Intawong[1], Samita Tanasarnsopaporn[2], Jakkapan Boonsritan[1], Zhe Sun[3], Chukkapong Comsup[4], Prapath Tienprateep[4], Zhaoqi Zhan[3] (Presenter)

1. Department of Forensic Medicine, Chulalongkorn University, Bangkok, BANGKOK, Thailand; 2. Department of Forensic Medicine, King Chulalongkorn Memorial Hospital, The Thai Red Cross Society, Bangkok, BANGKOK, Thailand; 3. Shimadzu (Asia Pacific) Pte Ltd, Singapore, Singapore, Singapore; 4. Bara Scientific Co., Ltd,, Bangkok, BANGKOK, Thailand

## 1. Overview

- Multi-target screening for 61 toxicological compounds in whole blood was established on a fully-automated platform consisting of CLAM and LC-MS/MS.
- Using the method package *Rapid Toxicology Screening*, tedious method development work was avoided.
- Co-injection to add pure water after sample pre-treatment on CLAM module can minimized solvent effect in the subsequent LC elution process.
- The Flag ID function of the LabSolutions Insight s/w was used to alert uncertainty such as large RT shift and unmatched ion ratio etc.

## 2. Introduction

Multi-target screening by LC/MS/MS has been widely adopted in detection and quantitation of drugs of abuse (DoA) in forensic investigation and toxicological research [1]. Usually, a wide range of targets are screened in such analysis, including illicit drugs, narcotics, psychotropics, antipsychotics, pharmaceuticals and other toxic compounds in urine, serum/plasma and whole blood samples. Sample preparation is often a bottleneck due to the tedious steps. It is also a factor responsible for inaccurate or false negative results. We describe a solution by using an automated sample preparation module CLAM-2000™ connected with LC/MS/MS system (LCMS-8060) for multi-target screening of 61 drugs in whole blood. A ready-to-use method package Rapid Toxicology Screening (Shimadzu) [2] was used to set up the screening method with human whole blood (frozen) spiked sample without efforts in LC and MRM method development.

## 3. Experimental

The 61 targeted drugs (see Table 2) with 26 deuterated drugs as internal standards (IS) were analyzed on a high throughput analysis platform, which consists of CLAM-2000™ coupling with the LCMS-8060 triple quadrupole system. Automated sample preparation process was carried out on the CLAM-2000 module involving pre-programed steps: wetting of filtering vial with solvent, blood sample dispensing (50uL), ACN dispensing (250uL), stirring for 60 seconds at 2000rpm, filtering for 90 seconds and vial transferring to autosampler. Co-injection (5uL sample + 20uL water) was performed for reducing solvent effect and improving the peak shape. The whole procedure was run automatically for a whole batch run including solvent, calibrants, blank, blood samples (spiked) and QCs.

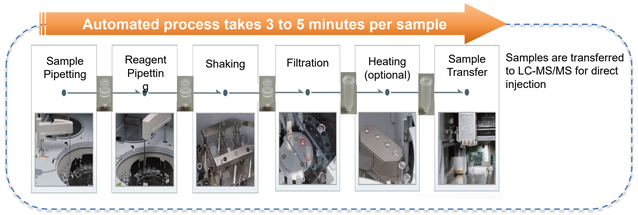


**Figure 1**: Workflow of CLAM-2000 for automated sample preparation coupled with LCMS-8060

**Table 1.** Analytical conditions for 61 toxicological compounds with 25 ISs on LCMS-8060

| | | | |
|---|---|---|---|
| **Column** | Kinetex XB-C18 (2.1 mm I.D. x 100 mmL., 2.6um) | **Interface** | ESI (heated) |
| **Flow rate** | 0.3 mL/min | **MS mode** | Positive, MRM |
| **Mobile phase** | A: 10mM of ammonium formate, with 0.1% formic acid<br>B: 10mM of ammonium formate, with Methanol and 0.1% formic acid | **Interface temp.** | 300°C |
| | | **DL temp.** | 250°C |
| | | **Heat block temp.** | 400°C |
| **Elution mode** | 0 min: 5% B → 2 min, 15% B → 10 min, 50% B → 12-20 min, 95% B → 20.1-26, 5% B (end) | **Nebulizing gas flow** | $N_2$, 3 L/min |
| | | **Drying gas flow** | $N_2$, 10 L/min |
| **Oven temp.** | 40°C | **Heating gas flow** | Air, 10 L/min |
| **Injection vol.** | 5.0 µL | | |

A clean human whole blood sample was thaw from deep frozen storage and was used as blank and the matrix for preparation of spiked samples for evaluation of the method performance. Following the method package LC conditions [2], the chromatographic separation of the drugs was achieved with a gradient elution in 26 minutes (See Table 1). Analysis of batch screening data was carried out using LabSolutions Insight version 3.5.

## 4. Results and Discussion

### 4.1 MRM method for analysis of 61 DoA

An MRM method with 2-3 optimized MRM transitions for each compound was used directly from the method package of Rapid Toxicology Screening (161 toxicological compounds) [2]. Retention times of the compounds were updated with standards. MRM optimization of some deuterated internal standards were carried out and the CE values were added the method. A chromatogram of the 61 target with 26 deuterated IS is shown in Figure 2.

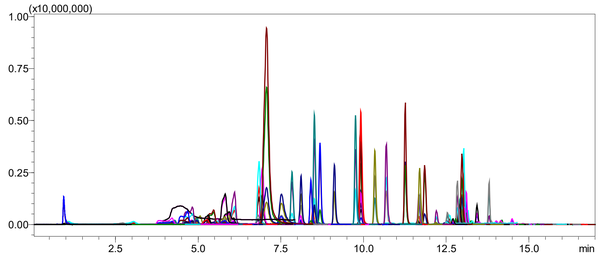


**Figure 2**: MS chromatogram of mixed standards of 61 DoA drugs (20 ppb) with IS at 4.0 ppb.

For the 61 targets, only 26 deuterated internal standards (D3~D10) were available. These 26 drugs each with IS are remarked with w.IS in Table 2. The other 35 compounds were screened and quantified with the respective IS which retentions are the closest to the targets. Linear calibration curves were established and good linearity with R2>/=0.99 was obtained for the 61 targets with three calibration levels of 4, 20 and 100 ppb and IS at 4 ppb (Table 2).

**Table 2.** Analytical conditions of screening of 61 DoA drugs analysis on LCMS-8060

| SN | Compound Name | Ret. Time (min) | MRM Quantifier (m/z) | ISTD Group | Calibration range (ppb) | $R^2$ |
|---|---|---|---|---|---|---|
| 1 | 6-Acethyl Morphine (w.IS) | 5.83 | 328.0>165.0 | 1 | 4-100 | 0.9961 |
| 2 | Amphetamine (w.IS) | 5.48 | 136.1>91.1 | 2 | 4-100 | 0.9968 |
| 3 | Benzoylecgonine (w.IS) | 7.53 | 290.2>168.2 | 3 | 4-100 | 0.9994 |
| 4 | Carbamazepine (w.IS) | 12.54 | 237.1>194.1 | 4 | 4-100 | 0.9972 |
| 5 | Clonazepam (w.IS) | 12.56 | 316.1>270.1 | 5 | 4-100 | 0.9982 |
| 6 | Cocaine (w.IS) | 8.51 | 304.2> 182.2 | 6 | 4-100 | 0.9995 |
| 7 | Alprazolam | 13.15 | 309.1>281.1 | | 4-100 | 0.9994 |
| 8 | Chlordiazepoxide | 12.61 | 300.1>227.1 | | 4-100 | 0.9996 |
| 9 | Clobazam | 12.89 | 301.1>259.1 | | 4-100 | 0.9996 |
| 10 | Dextromethorphan | 11.71 | 272.2>215.2 | | 4-100 | 0.9996 |
| 11 | Diazepam (w.IS) | 13.44 | 285.1>193.1 | | 4-100 | 0.9984 |
| 12 | Flunitrazepam | 12.70 | 314.1>268.1 | | 4-100 | 0.9994 |
| 13 | Flurazepam | 11.86 | 388.2>315.0 | 7 | 4-100 | 0.9980 |
| 14 | Lorazepam | 12.92 | 321.0>275.0 | | 4-100 | 0.9998 |
| 15 | Mescaline | 5.95 | 212.1>195.1 | | 4-100 | 0.9984 |
| 16 | Methylphenidate | 8.69 | 234.15>84.1 | | 4-100 | 0.9999 |
| 17 | Midazolam | 12.53 | 326.1>291.1 | | 4-100 | 0.9992 |
| 18 | Tramadol | 8.41 | 264.2>58.0 | | 4-100 | 0.9993 |
| 19 | Cannabinol | 13.86 | 311.2>222.9 | | 4-100 | 0.9933 |
| 20 | Anhydroecgonine methyl ester (w.IS) | 3.06 | 182.1>91.1 | 8 | 4-100 | 0.9984 |
| 21 | Estazolam (w.IS) | 12.93 | 295.1>267.1 | 9 | 4-100 | 0.9975 |
| 22 | Amitriptyline | 12.99 | 278.1>233.0 | | 4-100 | 0.9972 |
| 23 | Desipramine | 12.92 | 267.2>72.1 | 10 | 4-100 | 0.9941 |
| 24 | Imipramine (w.IS) | 12.85 | 281.2>86.1 | | 4-100 | 0.9938 |
| 25 | Trimipramine | 13.03 | 295.2>100.1 | | 4-100 | 0.9965 |
| 26 | MDA (w.IS) | 5.91 | 180.1>163.1 | 11 | 4-100 | 0.9961 |
| 27 | MDEA (w.IS) | 6.85 | 208.1>163.1 | 12 | 4-100 | 0.9966 |
| 28 | 3,4-Methylenedioxypyrovalerone | 9.13 | 276.2>126.2 | | 4-100 | 0.9960 |
| 29 | Cathinone | 4.30 | 150.1>117.1 | | 4-100 | 0.9983 |
| 30 | Fentanyl | 11.28 | 337.3>188.2 | | 4-100 | 0.9944 |
| 31 | Ketamine | 7.85 | 238.1>125.0 | | 4-100 | 0.9943 |
| 32 | LSD (Lysergic acid diethylamide) | 9.92 | 324.2>223.1 | 13 | 4-100 | 0.9937 |
| 33 | MDMA (w.IS) | 6.11 | 194.1>163.1 | | 4-100 | 0.9966 |
| 34 | Mephedrone | 6.95 | 178.1>145.1 | | 4-100 | 0.9971 |
| 35 | Methcathinone | 4.69 | 164.1>131.1 | | 4-100 | 0.9966 |
| 36 | Sibutramine | 13.11 | 280.2>125.1 | | 4-100 | 0.9945 |
| 37 | Methadone (w.IS) | 12.98 | 310.2>265.2 | 14 | 4-100 | 0.9966 |
| 38 | Methamphetamine (w.IS) | 5.83 | 150.2>91.1 | 15 | 4-100 | 0.9980 |
| 39 | Codeine (w.IS) | 4.98 | 300.2>152.1 | 16 | 4-100 | 0.9989 |
| 40 | Mitragynine (w.IS) | 11.69 | 399.1>173.9 | 17 | 4-100 | 0.9978 |
| 41 | Morphine (w.IS) | 2.72 | 286.15>152.10 | 18 | 4-100 | 0.9952 |
| 42 | Nalorphine | 4.82 | 312.10>201.00 | | 4-100 | 0.9901 |
| 43 | Naloxone | 4.98 | 328.15>212.10 | | 4-100 | 0.9923 |
| 44 | Naltrexone | 5.60 | 342.15>270.15 | | 4-100 | 0.9901 |
| 45 | Nimetazepam | 12.79 | 296.05>250.20 | 19 | 4-100 | 0.9940 |
| 46 | Nitrazepam | 12.50 | 282.10>236.10 | | 4-100 | 0.9952 |
| 47 | Nordiazepam (w.IS) | 13.26 | 271.05>140.05 | | 4-100 | 0.9964 |
| 48 | Pentazocine | 10.36 | 286.20>218.20 | | 4-100 | 0.9964 |
| 49 | Phencyclidine | 10.71 | 244.20>91.05 | | 4-100 | 0.9953 |
| 50 | Norpseudoephedrine (w.IS) | 4.21 | 151.95>134.05 | 20 | 4-100 | 0.9984 |
| 51 | Nortriptyline (w.IS) | 13.05 | 264.15>233.15 | 21 | 4-100 | 0.9913 |
| 52 | Oxazepam (w.IS) | 12.91 | 287.05>241.00 | | 4-100 | 0.9932 |
| 53 | Prazepam | 13.80 | 325.10>271.05 | | 4-100 | 0.9861 |
| 54 | Sildenafil | 12.67 | 475.20>58.05 | 22 | 4-100 | 0.9889 |
| 55 | Temazepam | 13.10 | 301.05>255.10 | | 4-100 | 0.9976 |
| 56 | Triazolam | 13.09 | 343.05>308.20 | | 4-100 | 0.9964 |
| 57 | Oxycodone (w.IS) | 5.32 | 316.15>241.15 | 23 | 4-100 | 0.9989 |
| 58 | R-Pseudoephedrine (w.IS) | 4.84 | 166.00>148.00 | 24 | 4-100 | 0.9976 |
| 59 | Zolpidem (w.IS) | 9.77 | 308.20>235.15 | 25 | 4-100 | 0.9991 |
| 60 | Zaleplon | 12.22 | 306.00>236.00 | | 4-100 | 0.9962 |
| 61 | Zopiclone (w.IS) | 8.12 | 389.10>244.95 | 26 | 4-100 | 0.9968 |

### 4.2 Quantitative screening of spiked whole blood sample

A whole blood sample free of the listed targets was thawed from deep frozen storage and used as the blank (added IS, 4 ppb) and matrix to prepare spike samples (added mix targets 10 ppb and IS 4 ppb) for determining recovery and and precision. A batch run was carried out automatedly on the CLAM-LC-MS/MS system, including solvent, blank (IS), calibrants (4, 20 and 100 ppb), spike samples (10 ppb) and QC sample (10 ppb).

Recovery and precision of this screening workflow were evaluated with 10 ppb spiked whole blood sample by determining for five times (n=5) on the CLAM-LC-MS/MS system. A representative chromatogram is shown in Figure 3. The recovery is calculated by: R(%) = [Area $_{\text{in spiked sample}}$ / Area $_{\text{in neat}}$) X 100%. The

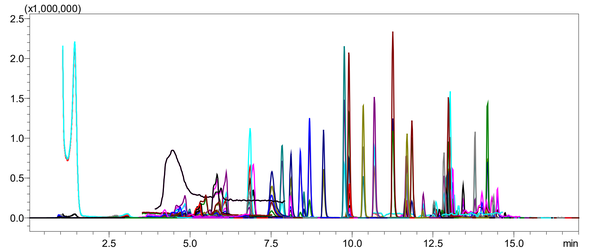


**Figure 3**: MS chromatogram of spike whole blood sample (target at 10 ppb with IS at 4.0 ppb) .

results indicate that recoveries of 53 targets out of 61 and 23 deuterated ISs out of 26 are within 70%~130%. The compounds which recoveries are out the range are 6-Acethyl Morphine (58.0%) and 6-Acethyl Morphine-D3 (32.3%); Carbamazepine (66.9%) and Carbamazepine-D10 (53.8%), as well as Midazolam (58.4%), Cannabinol (50.8%), Cathinone (41.3), Mephedrone (60.4), Methcathinone (45.2%), Nordiazepam (134.7%) and Morphine-D3 (152.2%). The precisions (RSD, n=5) of the above spiked samples are below 14.4% for the 61 targets and below 17.8% for the 26 internal standards.

It is worth to note that the Flag ID function of the LabSolutions Insight s/w was used in batch data analysis to alert large RT shift, unmatched ion ratio, poor linearity and accuracy etc., which provides a very efficient tool in reliability checking (Figure 4).

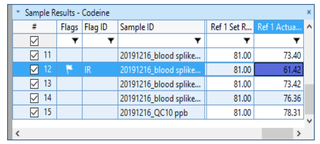


**Figure 4**: The "Flag ID" indicates the Ion Ratio of Codeine in a spike sample is out of the criteria (+/- 15% absolute).

## 5. Conclusions

A fully-automated platform consisting of CLAM-2000 and LC-MS/MS was used in establishing multi-target screening analysis for 61 toxicological compounds in whole blood samples. By using the method package Rapid Toxicology Screening, tedious method development work was avoided with only RT alignments and MRM optimization for some ISs. Co-injection with pure water after sample pre-treatment on CLAM module was found necessary to minimize solvent effect in the subsequent LC elution. LabSolutions Insight s/w was used in data analysis. The Flag ID function of the s/w was used to alert RT shift, unmatched ion ratio etc. This work demonstrates that the systems and s/w used could be helpful greatly in establishment of high throughput screening analysis for large numbers of targets in biological samples in toxicological research and investigation.

## Reference

1. Tiphaine Robin, Alan Barnes, Sylvain Dulaurent, Neil Loftus, Sigrid Baumgarten, Stéphane Moreau, Pierre Marquet, Souleiman El Balkhi and Franck Saint-Marcoux, Analytical and Bioanalytical Chemistry (2018) 410:5071–5083
2. Shimadzu Method Package - Rapid Toxicology Screening (Version 2), Shimadzu Corporation