Biochip array technology for morphine and benzodiazepine testing in urine of patients undertaking methadone maintenance therapy program

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Abstract. Forensic toxicology test includes a qualitative and quantitative test. Biochip array technology (BAT) is a new nanotechnological method employed in forensic toxicology. This study aimed to determine the effectiveness of BAT test in forensic toxicology of morphine and benzodiazepine in urine samples. This cross-sectional diagnostic study was applied to 20 patients aged >18 years in August 2014 at the primary health centers at Johar Baru, Jakarta Pusat. Gas chromatography (GC)/mass spectrometry (MS) of these samples identified four samples as morphine positive and three as benzodiazepine positive. BAT test identified four samples as morphine positive and six as benzodiazepine positive. Diagnostic analysis of morphine testing confirmed that BAT test showed 100% sensitivity, 100% specificity, 100% positive predictive value, and 100% negative predictive value. On the other hand, diagnostic test analysis of benzodiazepine testing confirmed that BAT test showed 100% sensitive, 82.35% specific, 50% positive predictive value, and 100% negative predictive value. Therefore, BAT test is reliable for morphine testing but only when the sample is controlled; however, it is unreliable for benzodiazepine testing.

1. Introduction

The misuse of narcotics and psychotropics has become a national threat in Indonesia. According to the Indonesia Constitution No. 35 in the year of 2009, narcotics can be defined as the “substance or medicine that comes from plant or non-plant, either synthetic or semisynthetic, which can cause the loss or alteration of consciousness, numbness of feeling, loss or even the elimination of pain, and can cause addiction, which can be grouped into several categories, as attached on this Constitution [1].” Data from Indonesian National Narcotics Agency until 2011 report that the narcotic user count had reached approximately 2.21% of the total population with individuals from diverse backgrounds [2]. Based on the data obtained from Jakarta Public Health Office in 2003, the most commonly used narcotics is heroin. In a forensic department, a forensic doctor must be able to diagnose an alleged case of poisoning specifically caused by narcotics [3]. The forensic laboratory test has two parts: qualitative test (screening) and quantitative test (confirmation) [4]. The qualitative test involves the use of the biochip array technology (BAT), which is based on the same principle as that of competitive enzyme-linked immunosorbent assay (ELISA). BAT test for toxicological examination have been performed in England and Italy. In Indonesia, this method was developed in 2013; however, it remains to be used for toxicology test. Past research based in England claims that this test has a sensitivity of 89.1% for...
morphine and of 78% for benzodiapine [5]. As per our knowledge, no research has yet been performed on the diagnostic score using this method in Indonesia. Therefore, we aimed to investigate the morphine and benzodiazepine diagnostic scores in urine samples using BAT test.

2. Methods
In this study, diagnostic test was performed according to a cross-sectional research design toward determining the diagnostic score of BAT test on individuals following the Rumatan Metadon Therapy Program in Johar Baru Community Health Center, Central Jakarta, during August 2014. The subjects were aged 19–47 years. This research was performed at the Johar Baru Community Health Center and Forensic and Medicolegal Department of Medicine Faculty, University of Indonesia, Jakarta. BAT test was performed in Forensic Laboratorium of Medicine Faculty, Universitas Indonesia. For the research purpose, a total of 16 urine samples were collected by consecutive sampling from local subjects who fulfilled the inclusion and exclusion criteria as mentioned below. Considering a 25% possibility of dropout, the total research samples subjected to testing was 20 [6].

The inclusion criteria for this research were subjects of either gender, of age >18 years, undergoing the Rumatan Metadon Therapy Program, and willing to participate in the study. The exclusion criteria for this research were unwillingness of the subjects to participate in the research and positive result for drugs other than morphine and benzodiazepine in the rapid screening test. The dropout criteria were erroneous sample test results using BAT test and discontinuation of subject participation in the middle of the research.

3. Results
Anamneses on the research subjects regarding morphine or benzodiazepine usage revealed that 8 and 4 subjects had previously consumed morphine and benzodiazepine, respectively (Table 1). The screening test for morphine using the Right Sign rapid test revealed positive morphine results in four samples (20%) and positive benzodiazepine result in six samples (30%).

| No | Age       | Sex | Narcotics history usage | Morphine test result with rapid test | Benzodiazepine test result with rapid test |
|----|-----------|-----|-------------------------|-------------------------------------|------------------------------------------|
| 1  | 36 years old | Male | None | Negative                | Negative                                |
| 2  | 46 years old | Male | None | Negative                | Negative                                |
| 3  | 26 years old | Male | None | Negative                | Negative                                |
| 4  | 35 years old | Male | None | Negative                | Negative                                |
| 5  | 28 years old | Male | None | Negative                | Negative                                |
| 6  | 31 years old | Male | None | Negative                | Negative                                |
| 7  | 40 years old | Male | None | Negative                | Negative                                |
| 8  | 33 years old | Male | Benzodiazepine | Negative | Positive                      |
| 9  | 28 years old | Male | Morphine (night) | Positive | Positive                      |
| 10 | 28 years old | Male | None | Negative                | Negative                                |
| 11 | 38 years old | Male | Benzodiazepine (morning) | Negative | Negative                      |
| 12 | 30 years old | Male | None | Negative                | Negative                                |
| 13 | 29 years old | Male | Morphine (morning, 9 o’clock) | Negative | Negative                      |
| 14 | 29 years old | Male | Morphine (morning) | Negative | Negative                      |
Table 1. Continue

| No | Age       | Sex  | Narcotics history usage | Morphine test result with rapid test | Benzodiazepine test result with rapid test |
|----|-----------|------|-------------------------|-------------------------------------|-------------------------------------------|
| 15 | 33 years old | Male | Morphine (morning)      | Positive                            | Negative                                  |
| 16 | 32 years old | Female | Morphine (morning, jam 10) | Positive                            | Negative                                  |
| 17 | 47 years old | Male | Morphine (morning, 10.30) | Positive                            | Negative                                  |
| 18 | 25 years old | Male | Morphine (2 days ago) with sucking method benzodiazepine (night) | Negative                            | Negative                                  |
| 19 | 35 years old | Male | Morphine (2 years ago)  | Negative                            | Positive                                  |
| 20 | 19 years old | Male | MDMA (night ago) benzodiazepine (night ago) | Negative | Positive |

Table 2. Morphine test using Biochip Array Technology (BAT) test and GC/MS

| Sample no. | Test result with BAT | Test result with GC/MS | Morphine concentration using the BAT test (ng/ml) | Morphine concentration using the GC/MS (ng/ml) |
|------------|----------------------|------------------------|---------------------------------------------------|---------------------------------------------|
| 1          | Negative             | Negative               | -                                                  | -                                           |
| 2          | Negative             | Negative               | -                                                  | -                                           |
| 3          | Negative             | Negative               | -                                                  | -                                           |
| 4          | Negative             | Negative               | -                                                  | -                                           |
| 5          | Negative             | Negative               | -                                                  | -                                           |
| 6          | Negative             | Negative               | -                                                  | -                                           |
| 7          | Negative             | Negative               | -                                                  | -                                           |
| 8          | Negative             | Negative               | -                                                  | -                                           |
| 9          | Positive             | Positive               | >2804                                              | 2830                                        |
| 10         | Negative             | Negative               | -                                                  | -                                           |
| 11         | Negative             | Negative               | -                                                  | -                                           |
| 12         | Negative             | Negative               | -                                                  | -                                           |
| 13         | Negative             | Negative               | -                                                  | -                                           |
| 14         | Negative             | Negative               | -                                                  | -                                           |
| 15         | Positive             | Positive               | >2804                                              | 42690                                       |
| 16         | Positive             | Positive               | >2804                                              | 38000                                       |
| 17         | Positive             | Positive               | >2804                                              | 28760                                       |
| 18         | Negative             | Negative               | -                                                  | -                                           |
| 19         | Negative             | Negative               | -                                                  | -                                           |
| 20         | Negative             | Negative               | -                                                  | -                                           |
The morphine test using BAT test revealed positive results for six samples (30%). These samples were then re-tested for confirmation by gas chromatography (GC)/mass spectrometry (MS) to reveal that only three of the six samples were positive for morphine (15%). The comparison of the test results using BAT test and GC/MS is shown in Table 2.

Table 3. The diagnostic test result of morphine test with biochip array technology in urine samples of patients undertaking the Rumatan Metadon Therapy Program.

| GCMS for morphine | BAT for morphine |
|-------------------|------------------|
| Positive          | Positive         |
|                   | 4                |
|                   | 0                |
|                   | 4                |
| Negative          | Negative         |
|                   | 0                |
|                   | 16               |
|                   | 16               |
|                   | 4                |
|                   | 16               |
|                   | 20               |

\[ \text{df} = 1; \ p = 0.000 \ (\text{Fisher Test}) \]

As per Table 3, the instrument sensitivity for urine samples was 100%, with the highest specificity of 100%, the positive predicted value (PPV) of 100%, the negative predicted value (NPV) of 100%, and accuracy of 100%. The area under the ROC curve to test for morphine using BAT test was 1 (95% CL: 1–1).

Table 4. The diagnostic test result for benzodiazepine with biochip array technology in urine samples from patients undertaking the Rumatan Metadon Therapy Program.

| GCMS for benzodiazepin | BAT for benzodiazepin |
|------------------------|-----------------------|
| Positive               | Positive              |
|                        | 3                     |
|                        | 3                     |
|                        | 6                     |
| Negative               | Negative              |
|                        | 0                     |
|                        | 14                    |
|                        | 14                    |
|                        | 3                     |
|                        | 17                    |
|                        | 20                    |

\[ \text{df} = 1; \ p = 0.018 \ (\text{Fisher test}) \]

As per Table 4, the instrument sensitivity using the urine sample was 100%, with the highest specificity of 82.35%, PPV of 50%, NPV of 100%, LR+ of 5.67, LR− of 0, and accuracy of 85%. The area under the ROC curve on the benzodiazepine using BAT test was 0.912 (95% CL: 0.782–1).

4. Discussion

The detection of narcotics using BAT test involves a biochip technology that allows multi-analytical test with the competitive ELISA principle. This method has been in application in 22 countries, including Indonesia. However, this method has rarely been used in toxicology test. The present research was aimed at investigating whether BAT test can be used for sensitive or specific narcotics test.

This study obtained a morphine sensitivity of 89.1% by morphine testing using BAT test, which showed 100% sensitivity and specificity. The infinite positive probability of morphine using BAT test ascertains the reliability of this testing method. With PPV and NPV of 100% each, BAT test can be said to show an excellent ability of detecting the true positive or negative result.

As per the research by Randox Laboratory, for benzodiazepine testing, the sensitivity was 78%. As per the BAT test, for benzodiazepine testing, the sensitivity was 100%, specificity was 82.35%, PPV was 0.5 (50%), NPV was 1 (100%), LR+ was 5.67, and LR− was 0. PPV obtained for the benzodiazepine
test using BAT test indicates the efficiency of this testing method to provide the true positive result of 50% and the true negative result of 100%; these findings together confirm that this method is not adequate for the detection of benzodiazepine.

The false positive score obtained in this research for three samples (samples #8, 9, and 19) can be attributed to the use of oxaprozin and ceraltrine drugs [7]. Based on the research by Antje et al., the use of anti-retro viral therapy can also result in false positive result in benzodiazepine screening test [8].

The ratio of PPV obtained in morphine testing was infinity, while that of NPV was 0. With the ratio of infinitive PPV, the post-test probability value was increased, thereby confirming that the result obtained in morphine testing using BAT test was appropriate. Moreover, the area under the curve in this research was 100%, thereby confirming that morphine testing using BAT test was accurate. In the benzodiazepine test, the ratio of PPV was 5.67, while that of NPV was 0. The value for pre-test probability score was set at 80%. With PPV of 5.67, the post-test probability score was increased to 95.78%. The post-test probability score ascertained the researcher’ belief regarding the reliability of the detection of benzodiazepine using BAT test. The area under the ROC curve on the benzodiazepine test using this method was 91.2%, which leads to the conclusion that BAT test can help in the detection of benzodiazepine with the probability of 91.2%.

In morphine testing, a total of four samples were found to be positive. The subsequent concentration test of these four samples using BAT test revealed a value of >2804 ng/mL. Furthermore, the results of these tests was confirmed by GC/MS, which estimated the values of 2.83, 42.69, 38, and 28.76 ng/mL. The difference in the values obtained by the morphine testing using BAT test and GC/MS was huge. The commonly consumed morphine dosage (therapeutic dosage/non-toxic) was 5–30 mg every 4–8 h. The therapy dosage concentration (non-toxic) that was founded on morphine test with the urine sample was 0.5–20 mg/L (500–20,000 ng/mL), whereas the lethal dosage concentration of morphine detected in the urine samples was 11–323 mg/L (11,000–323,000 ng/mL). The test result obtained with BAT test was quite high, albeit within the therapeutic dosage concentration limitation. On the other hand, the results obtained with GC/MS was below the therapeutic dosage concentration.

In benzodiazepine testing using BAT test, six samples were positive, with the concentration level of 557 ng/mL (sample #8, 11, 13, and 20), 252 ng/mL (sample #9), and 266.5 ng/mL (sample #19), whereas GC/MS identified only three positive samples, with the concentration level of 210 ng/mL (sample #11), 790 ng/mL (sample #13), and 420 ng/mL (sample #20). The lethal dosage concentration of benzodiazepine, particularly alprazolam, in the urine sample was 1 mg/L (1000 ng/mL) [7]. In the three samples showing positive results, the concentration level were below the benzodiazepine lethal dosage value.

This research has many limitations that may have affected the results. Due to the lack of research funding, the total number of samples analyzed were insufficient for appropriate total sample calculations. In addition, a possibility of cross-reactivity between benzodiazepine with the anti-retro viral drugs could not be confirmed using the standardized method because of the lack of the standard drug efavirenz. The false positive result obtained to in the benzodiazepine result, which possibly occurred because of the cross-reactivity reaction to anti-retro viral drugs, was only based on the medical record data to the research sample.

In a previous research conducted in the Randox Laboratorium in England, the possibility of the cross-reaction of codein test to morphine was found to be 69.5%. In this research, the samples used came from the controlled population including the users of heroine/morphine following the Rumatan Metadon Therapy Program, with a known usage history of morphine or heroine; therefore, after the confirmation test, the presence of codein was not found. Finally, as the usage of codeine is presently not permitted as a medicine, it is difficult to prove the presence of cross-reaction between the morphine usage and codein.

5. Conclusion
The morphine testing method using BAT showed excellent sensitivity and specificity, suggesting its application for true positive and negative testing for morphine. The benzodiazepine testing method using BAT also showed excellent sensitivity, but good specificity, and no ability to detect true positive result
from the poor positive test results. However, this method showed a 100% capability for detecting benzodiazepine with the true negative result.

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