Comparison of Sensitivity and Specificity of Commercial Amphetamine Tests

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Abstract: Drug addiction is a disease that is characterized by a compulsion, a desire to take different substances permanently or for a certain period of time. Numerous negative incidents, such as crimes, work accidents and traffic accidents, are related to using illegal substances. Therefore, urine drug cassette tests have become a screening tool. However, considering legal consequences of test result, the question arises of their performance and reliability. On this account, the main objective of this study was to evaluate the sensitivity and specificity of urine drug tests available on the commercial pharmaceutical market. Evaluated tests were immersed in synthetic urine diluent spiked with amphetamine at various concentrations also containing potentially interfering substances such as caffeine, paracetamol and acetylsalicylic acid, and after a certain period of time, it was observed whether the result was as expected. The reference method used in this study was high-performance liquid chromatography. The obtained results confirmed the declared cut-off as well as specificity of rapid diagnostic tests.

Keywords: amphetamines; cassette tests; high-performance liquid chromatography; rapid drug detection test

1. Introduction

According to the latest data, drug addiction is very common among people aged 15–69, of whom as many as 5.9 million men and 2.7 million women have used amphetamine at least once in their lives [1]. Amphetamine is a potent central nervous system stimulant [2] and represents a class of psychotropic compounds commonly abused for their stimulant, euphoric, anorexic and in some cases empathogenic and hallucinogenic properties [3]. Initially, amphetamine was investigated for a drug substance. In 1929, the first racemic test on humans was performed, and in the following years, studies were carried out in London, in which as many as 150 people, mainly women, took part [4]. At that time, amphetamine in low doses was shown to increase cognitive abilities, and tablets with this substance were gaining popularity among stimulant drugs [5]. Even during World War II, amphetamines were given to soldiers to relieve fatigue. However, this drug began to be abused by combatants, and doctors decided to classify the substance as a narcotic [5,6].

The first rapid cassette tests appeared already in the 1980s, but they were not characterized by satisfactory sensitivity, which improved in the following years [7]. Rapid immunochemical drug tests available on the pharmaceutical market cover basic, classic narcotics and some drugs. New additive substances are constantly being developed in illegal laboratories, many of them appearing locally or seasonally and disappearing with changing fashions. This means that the available drug test panel covers only some of the psychoactive substances used, and a negative result does not mean that the patient is not under the influence of another substance (synthetic or natural). In such cases, a discrepancy between the clinical condition of the patient with symptoms of the drug effect and
negative test results should be taken into account. Blood, saliva, urine or even fingernails can be used to test for the presence of drugs in the body. The choice of the appropriate biological material depends on the purpose of the test. Urine is the most suitable material for controlling drug abstinence and diagnosing acute conditions. Moreover, urine is the most frequently used test material, mainly due to the ease of collection but also the highest concentration of drugs or drug metabolites, i.e., a larger detection window than, for example, in the case of blood serum concentration. It should be taken into account that people addicted to drugs and controlled for drug abstinence may use various methods to falsify the result of the toxicological analysis. Various practices are used: dilution of urine, addition of foreign substances or substitution of the sample. The most common is dilution by adding water or other fluids to the urine sample. Dilution may also be the result of increased fluid intake or the use of diuretics several hours before the scheduled examination [8]. The undoubted advantage of immunochemical tests is the possibility of using them as a negative screening technique and rule out an individual specimen that requires no further analytical work to be performed. The routine use of rapid tests can help to reduce the resources and running costs associated with drug testing as well as to increase the throughput of laboratories where more sensitive analyses can be limited only to positive samples requiring confirmation of the presence of the tested substance and obviously to shorten the waiting time for the result [9].

The aim of the study was to assess the sensitivity and specificity of drug immunochemical cassette tests available on the commercial pharmaceutical market for the detection of amphetamine, taking into account the possibility of false-positive or false-negative results.

2. Materials and Methods

In the present study following reagents were used: standardized solution of dextro-amphetamine at a concentration of 1 mg/mL in methanol, certified reference material (Merck, Darmstadt, Germany; CAS number: 51-64-9), monobasic sodium phosphate dihydrate (>99%) (Merck, Darmstadt, Germany), paracetamol (98–102%) (Merck, Darmstadt, Germany), caffeine (98.5%) (Thermo Scientific Chemicals, Walham, MA, USA) and acetylsalicylic acid (99.5%) (Merck, Darmstadt, Germany), Sigmatrix Urine Diluent (Merck, Darmstadt, Germany), hydrochloric acid 35–38% (Avantor Performance Materials Poland S.A., Gliwice, Poland) and acetonitrile (Merck KGaA, Darmstadt, Germany). Water was prepared using the Milli-Q Water Purification System (Millipore, Molsheim, France).

Experiments were carried out on a Shimadzu high-performance liquid chromatography set (Kyoto, Japan) equipped with a vacuum degasser (DGU-20A5), a solvent pump (LC-20AD), an autosampler (SIL-20AD), a diode array detector (SPD-M20A), a column oven (CTO-20AC), a communications bus module (CBM-20A) and LC Solution software ver. 1.0.0.1 (Shimadzu Corporation, Kyoto, Japan).

For the qualitative and quantitative analysis of amphetamine in synthetic urine diluent in the possible presence of caffeine, acetylsalicylic acid and paracetamol, the method described by F. Sadeghipour et al. [10] was applied after validation, performed according to ICH guidelines [11].

For chromatographic conditions: the mobile phase was composed of 20 mM NaH₂PO₄ × 2H₂O and acetonitrile in a ratio of 91:9. The solid phase was C18 (octadecyl), i.e., Kinetex® C18 HPLC Column100 (100 mm × 4.6 mm; particle size, 2.6 µm; pore size, 100 Å) was used. The temperature was set to 40 degrees, and the pressure was 263 bar. The average retention time was 5.13 min. The samples were dissolved in 0.1 M HCl, and the injection was 20 µL. The photodiode array detector was set to 200 nm.

The test material consisted of solutions of amphetamine in synthetic urine diluent at various levels of concentration, imitating samples from a patient. Caffeine, aspirin and paracetamol solutions in synthetic urine diluent as well as amphetamine and caffeine solutions in synthetic urine diluent were also used. In order to assess sensitivity and specificity, the examined cassette tests were immersed in abovementioned solutions in triplicate. Amphetamine solutions with the declared concentrations of 10, 5, 1, and 0.5 µg/mL were
prepared to assess the sensitivity of rapid drug tests. In order to confirm their actual concentrations, they were subjected to quantitative analysis using the abovementioned chromatographic method. The qualitative analysis also included solutions of paracetamol, caffeine and acetylsalicylic acid at a concentration of 150 µg/mL in synthetic urine diluent to assess the possibility of a false-positive cassette test result, as well as solutions of amphetamine with caffeine at a concentration of 1 and 150 as well as 5 and 150 µg/mL, respectively, to assess the possibility of a false-negative result in the plate test. The selectivity of the method was tested by injecting samples containing of amphetamine and possible adulterants (paracetamol, caffeine and acetylsalicylic acid) at a concentration of 10 µg/mL separately and together as a one solution at the same concentrations. Calibration curves were made for amphetamine at concentrations between 0.50 and 20.0 µg/mL (0.50, 5.0, 10.0, 15.0 and 20.0 µg/mL). Experimental values were plotted as a function of theoretical values. Standard calibration curves were obtained from unweighted least squares linear regression analysis. The linearity of the method was statistically verified. Confidence intervals were calculated for intercept and slope (Student’s t-test, 95% confidence level).

3. Results

High-performance liquid chromatography in the work was treated as a reference method because determinations made using rapid tests are only of a screening nature, and the results should be verified in toxicology laboratories using reference methods.

3.1. Validation of a Chromatographic Method for the Determination of Amphetamine in Synthetic Urine Diluent

The determined validation parameters for the developed chromatographic method are presented in Table 1, where \( r^2 \) is the correlation coefficient, \( y^- \) is the measured value; \( a^- \) is slope coefficient (a measure of sensitivity of the analytical method), \( x^- \) is amount of analyte and \( b^- \) is the shift of the regression line [12].

<table>
<thead>
<tr>
<th>( r^2 )</th>
<th>( Y = ax + b )</th>
<th>Accuracy</th>
<th>Repeatability</th>
<th>Intermediate Precision</th>
<th>Limit of Detection (LOD) (µg/mL)</th>
<th>Limit of Quantification (LOQ) (µg/mL)</th>
<th>Measurement Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9999</td>
<td>148,495.7251</td>
<td>5852.5099</td>
<td>100.12 ± 0.15%</td>
<td>0.13%</td>
<td>0.36%</td>
<td>0.13</td>
<td>0.40</td>
</tr>
</tbody>
</table>

3.1.1. Specificity

Chromatographic analysis of the blank matrix (synthetic urine diluent) and the matrix with the tested compound (amphetamine) confirmed the absence of additional matrix components during amphetamine retention. This means that the synthetic urine diluent components did not affect the results of the amphetamine assay in any way (Figures 1 and 2).

During the research, it was shown that the developed method also enables the identification and quantitative determination of amphetamine in the presence of caffeine, paracetamol and acetylsalicylic acid, which, according to literature data [7] and information available in strip test packages, may cause a false-positive test result for the presence of amphetamine in urine. These substances were in no way a source of signal interference, which is clearly visible on the chromatogram (Figure 3).

3.1.2. Linearity

Linearity was confirmed by the calculation a calibration curve for five amphetamine standard solutions in synthetic urine diluent at the following concentrations: 0.5, 5.0, 10.0, 15.0 and 20.0 µg/mL. The significance of the slope coefficient, the intercept and the value of the correlation coefficient clearly indicate a linear interdependence (Table 1).
During the research, it was shown that the developed method also enables the identification and quantitative determination of amphetamine in the presence of caffeine, piroxicam and acetylsalicylic acid, which, according to literature data [7] and information available in strip test packages, may cause a false-positive test result for the presence of amphetamine in urine. These substances were in no way a source of signal interference, which is clearly visible on the chromatogram (Figure 3).

### 3.1.3. Accuracy

The accuracy of the method was expressed as percentage recovery by adding a known amount of analyte to the test sample. It was determined on the basis of triple determinations for three concentration levels. In the case of the main component, the recovery should be 80–110% [13]. During the tests, an accuracy of 100.12 ± 0.15% was obtained, which meets the above acceptance criteria.
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3.1.4. Repeatability and Intermediate Precision
Precision was assessed using nine determinations covering the specified range for the procedure (three concentrations/three replicates each). The intermediate precision was evaluated with an interval of three days. In order to assess the repeatability, the value of the relative standard deviation (RSD) was used, expressed as the CV(%RSD) coefficient of variation, which in the study at individual concentrations ranged from 0.28 to 1.26. It is worth noting that for the main component it should not exceed 11% [13]. The results show that the precision is acceptable. The intermediate precision for this method is as low as 0.36%.

3.1.5. Limit of Detection and Limit of Quantification
In the present study, the limit of detection (LOD) was 0.13 µg/mL. The LOD is the smallest substance concentration that can be detected by a given method. For comparison, the detection limit for rapid drug tests is 1 µg/mL. It should be noted that this value is lower than the cut-off of the test subjects, which confirms the choice of elaborated HPLC method as a reference method for the determination of amphetamine in synthetic urine diluent. The limit of quantification (LOQ), i.e., the lowest concentration of the test substance in the sample that can be quantitatively determined with appropriate precision and accuracy, was 0.40 µg/mL in the present work, which allows the determination of amphetamine in a sample even below the cut-off value of immunochemical tests for detecting drugs.

3.1.6. Measurement Range
The measurement range corresponds to the concentrations used to prepare the calibration curve, i.e., 0.5–20.0 µg/mL. At these concentrations, the developed method is characterized by acceptable linearity, accuracy and precision.
3.1.7. Quantitative Analysis of Amphetamine in Samples to Evaluate the Sensitivity of Cassette Test

In order to determine the actual concentrations of solutions prepared for the evaluation of the sensitivity of immunochemical tests for the detection of amphetamine in synthetic urine diluent, they were subjected to HPLC analysis as described in Section 2. The actual concentrations of amphetamine solutions were as follows: 0.45, 0.92, 4.95 and 9.92 µg/mL. The results are presented in Table 2.

Table 2. Determination of amphetamine in synthetic urine diluent samples.

<table>
<thead>
<tr>
<th>Declared Contents (µg/mL)</th>
<th>Tagged Content (µg/mL)</th>
<th>Recovery (%)</th>
<th>Mean Recovery (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.46</td>
<td>91.29</td>
<td>90.32</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td>90.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td>89.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.92</td>
<td>92.26</td>
<td>92.00</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>0.93</td>
<td>92.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.91</td>
<td>91.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>4.95</td>
<td>99.04</td>
<td>99.10</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>4.94</td>
<td>98.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.97</td>
<td>99.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>9.94</td>
<td>99.36</td>
<td>99.16</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>9.93</td>
<td>99.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.88</td>
<td>98.84</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.1.8. Evaluation of Sensitivity and Specificity of Cassette Tests

For all tests used in the study, a positive result is indicated by the presence of one strip in the test field.

All assessed tests unequivocally confirmed the presence of amphetamine in a synthetic urine diluent sample at a concentration of 9.92 µg/mL. Only one strip clearly appeared in all test windows. Moreover, the chromatogram revealed a peak in the fifth minute of the analysis, indicating the presence of amphetamine (Table 3).

Table 3. Results obtained for rapid drug tests and HPLC analysis for amphetamine samples with a declared concentration of 10 µg/mL.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Type of Test</th>
<th>Test a</th>
<th>Test b</th>
<th>Test c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamine 10 µg/mL</td>
<td>Concentration/HPLC</td>
<td>Result: Positive</td>
<td>Result: Positive</td>
<td>Result: Positive</td>
</tr>
<tr>
<td></td>
<td>result: 9.92 µg/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Precision: 0.28%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Cont.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Type of Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamine 10 µg/mL Test</td>
<td>Positive</td>
</tr>
<tr>
<td>Precision: 0.28%</td>
<td></td>
</tr>
<tr>
<td>Concentration/ HPLC result: 9.92 µg/mL</td>
<td></td>
</tr>
</tbody>
</table>

A similar situation occurred in the case of the next tested solution at a concentration 4.95 µg/mL. All evaluated tests unequivocally confirmed the presence of amphetamine in the synthetic urine diluent sample. Only one strip clearly appeared in the test windows, and a peak indicating the presence of amphetamine appeared in the chromatogram in the fifth minute of the analysis. For the succeeding concentration used for the cassette test assessment, HPLC analysis revealed the actual concentration of 0.92 µg/mL, which is slightly lower than the declared cut-off value. Therefore, it is not surprising that drug panel tests intended for the detection of several psychoactive substances was negative (two stripes). However, the amphetamine cassette test returned a positive result—only one strip clearly appeared in the test field. The chromatogram showed a peak at the fifth minute, indicating the presence of amphetamine in the sample. Similarly, for the last used concentration of 0.45 µg/mL (below the cut-off value), panel tests were negative, so two strips appeared in the test window. Interestingly, in the case of the amphetamine cassette test, one strip appeared in the test window, confirming a positive result (Table 4). This demonstrates the very high sensitivity of this test. A peak appeared on the chromatogram in the fifth minute of the analysis, indicating the presence of amphetamine in the sample (Table 4).

Each package of a rapid drug test is accompanied by information about substances that may interfere at a concentration greater than 100 µg/mL and thus falsify the test result. When testing samples with paracetamol, caffeine and acetylsalicylic acid at a concentration exceeding the abovementioned level, all tests unequivocally returned negative results. The exemplary results in the case of paracetamol are presented in Table 5. In this way, it was confirmed that these substances did not interfere with and did not cause a false-positive result in amphetamine immunochemical tests. Despite the use of substances in concentrations that, according to the manufacturers’ indications, may potentially negatively affect the specificity of the test, such an effect was not observed (Table 5).
Table 4. Results obtained for rapid drug tests and HPLC for amphetamine samples with a declared concentration of 0.5 µg/mL.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Type of Test</th>
<th>Concentration/HPLC result: 0.45 µg/mL</th>
<th>Precision: 1.26%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamine 0.5 g/mL</td>
<td>Test a</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Test b</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Test c</td>
<td>Negative</td>
<td></td>
</tr>
</tbody>
</table>

The second sample contained amphetamine at a concentration of 0.5 g/mL, which is equal to the cut-off value, and caffeine at a concentration of 150 µg/mL. In the first case, the obtained results were in agreement with the phenomenon described above. Namely, the panel tests were negative, and the test detecting only amphetamine gave a positive result. In the second case, all tested tests confirmed the presence of amphetamine in the synthetic urine diluent sample, i.e., the influence of caffeine on test results was not observed.
Table 5. Rapid drug test results for a 150 µg/mL paracetamol sample.

<table>
<thead>
<tr>
<th>Substance Type of Test</th>
<th>Substance Type of Test</th>
<th>Substance Type of Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol</td>
<td>Test a</td>
<td>Test b</td>
</tr>
<tr>
<td>150 µg/mL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Result:</th>
<th>Result:</th>
<th>Result:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Chromatogram

4. Discussion

Psychoactive substances are increasingly used around the world. The gravity of the problem has been reflected in criminal statistics. For instance, in the USA as many as 26% of all arrests are related to drug offenses. Moreover, as many as 1.16 million Americans are arrested annually for the sale, manufacture or possession of illegal substances [14]. Drug use in the workplace also has become a matter of concern. In order to ensure the safety of the workplace, employers want to determine whether their employees use illegal substances [15]. Drug testing is also a routinely performed in drug rehab centers not only at admission but also during the recovery treatment to make sure an individual patient is drug-free. If not, the patient is ordered to undergo detoxification [16]. In all abovementioned cases, accurate and quick results of a drug test are essential.
Considering the legal consequences of a drug test result, rapid drug tests are expected to have best possible sensitivity and specificity. The possibility of a false-positive or false-negative test result should be reduced to a minimum. In the light of this, the aim of the present study was to evaluate these parameters. The method of chromatographic determination of amphetamine in synthetic urine diluent elaborated by F. Sadeghipour et al. [10] was adopted, validated and finally used as a reference method in the present study.

Analysis of the obtained validation parameters legitimizes the right choice of HPLC method as a reference. In the present study, it was utilized for qualitative and quantitative purposes, i.e., it confirmed the presence of amphetamine in synthetic urine diluent solution as well as indicated the actual concentration of a particular solution used for the cassette test assessment. The accuracy of the HPLC method was 100.12 ± 0.15%, which meets the acceptance criteria because the expected recovery as a function of analyte concentration for the case of amphetamine determination according to AOAC International Guidelines for Standard Method Performance Requirements should be between 80–110% [13]. Acceptable precision was also achieved, expressed in terms of imprecision as a percentage of the relative standard deviation, which does not exceed 11%, which agrees with the cited source and ICH guidelines. The limit of detection was 0.13 µg/mL, and the limit of quantification was 0.40 µg/mL, which is below the literature cut-off values in urine for amphetamine [11]. The specificity of the method used was also tested, which allowed the determination of amphetamine with the simultaneous presence of caffeine, acetylsalicylic acid and paracetamol (as substances cross-interfering with the determination of amphetamine) in the sample.

The performed sensitivity assessment definitely speaks for the high quality of the rapid drug tests available on the pharmaceutical market. The analysis showed that all tests clearly showed a positive result above the literature cut-off value, i.e., 1 µg/mL [7]. Rapid tests intended for detecting one drug were positive even below this value, which shows that the sensitivity of these tests is even higher than declared. There are also tests available on the world market for which the cut-off value declared by the manufacturer is 0.5 µg/mL [17]. The tests are also inexpensive and quick to perform, and as a result they are quite common in the medical environment [18]. They are often used in clinical, professional, educational and legal settings [17]. Another advantage is the fact that drug tests can be performed on various biological samples, such as urine, blood, hair, saliva, sweat, nails and even meconium. Not only are pure psychoactive substances detected in the samples but also their metabolites [17]. Drug testing is also sometimes used to control the use of drugs, such as opioid analgesics, and to prevent abuse of these substances [19].

In most cases drug tests are specific, and the number of potentially interfering substances is relatively low; however, amphetamine is an exception, for which the largest number of interfering substances was recorded [17,20]. For these reasons, the assessment of the specificity of the immersion tests was an important part of current study, even if it was conducted only for three different compounds. For this purpose, solutions of acetylsalicylic acid, paracetamol and caffeine, i.e., substances potentially interfering with the determination of amphetamine, were prepared [7,17]. All evaluated tests gave unequivocally negative results for the presence of amphetamine. This means that the presence of the abovementioned substances in a urine sample does not increase the risk of a false-positive result. Caffeine in combination with amphetamine also did not affect the test result, i.e., no false-negative result was recorded. However, there are many interfering substances, and it should be remembered that this study did not fully confirm the ideal specificity of immunochemical tests because cases of “false-positive” results after ingestion of metformin [21] or benzathine salt of phenoxy methylpenicillin [22] have been described. The list of potentially interfering substances is long, and the study presented results only for three substances that may potentially be taken by patients; therefore, confirmation of a positive test result by a reference method should be considered. Although the quality of the current commercial tests is high compared to the first ones on the market, and the test execution is simple, the interpretation of the obtained results still remains complicated. This is due to the limited specificity of the immunological methods on which the tests are based. This can lead to
generating false-positive and false-negative results, which affects not only patient management but also potential legal consequences in the case of false positives. Therefore, for all samples determined by rapid tests as positive, it is recommended to perform confirmatory testing with higher-specificity techniques such as GC-MS or LC-MS. In justified cases, execution of confirmatory testing is also recommended for positive and negative results. In practice, no research confirmatory tests are routinely carried out, as they involve additional costs, the need to send the material to a toxicology laboratory and extended waiting time for the result. Therefore, in the absence of confirmation, the rapid test’s result should be commented on accordingly. Given the limitations of rapid immunoassays, it is advisable to provide information on the type of commercial test used (trade name, manufacturer), test sensitivity (cut-off) and possible interferences. An additional comment should relate to the detection window characteristic of the substance to be determined, toxicokinetics and individual factors (e.g., age, diet, organ performance, medications or stimulants taken, duration of addiction and tolerance) [7].

5. Conclusions

The present contribution confirms the satisfactory sensitivity and specificity of rapid immunochemical drug tests. The assessed parameters are not only in line with the declarations of manufacturers, but also, in some cases, the sensitivity is higher than expected.

Author Contributions: Conceptualization, A.M. and M.G.; methodology, A.M., M.G., P.K. and A.P.; software, P.K. and M.K.; validation, M.G.; formal analysis, A.P., A.M. and M.G.; investigation, A.M. and A.P.; resources, M.K.; data curation, M.G. and P.K.; writing—original draft preparation, A.M. and M.G.; writing—review and editing, M.G., A.P. and P.K.; visualization, A.M., A.P. and M.G.; supervision, M.G. and M.K.; project administration, M.G.; funding acquisition, M.G. All authors have read and agreed to the published version of the manuscript.

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