ISSN: 2717-8161 RESEARCH ARTICLE

New Trend Med Sci 2022; 3(2): 91-97. https://dergipark.org.tr/tr/pub/ntms

Strategy for Successful Urine Sample Preparation for LC-MS/MS Device at Drug Verification Laboratory

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Article History
Received 05 Dec 2020
Accepted 08 March 2022
Published Online 16 Sep 2022

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DOI: *10.56766/ntms.823790*

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Abstract: The use of liquid chromatography with tandem mass spectrometry (LC-MS/MS) device has increased significantly in toxicology validation laboratories in recent years. The maintenance cost of this expensive equipment is high as well as the cost of consumables. Reduction of the matrix effect and preparation more clear samples are very important for the validation of methods in clinical laboratories. The primary goal is to create a cleaner urine sample preparation technique to reduce the cost of maintenance of the LC-MS/MS device without affecting test results. We prepared the patients' urine in two different ways; routine urine preparation method and used our centrifuged method (14000 rpm, 10 minutes) for routine illicit substance use. The standard material used to determine whether there was a statistical difference in the urine sample with both different methods was added to both urine samples. Our findings showed that there was no statistical difference between the results of both methods for detection of illicit substance use. There was no difference between the high and low quantities of the 14 illicit substances measured and the centrifuged method and routine urine preparation methods ($p>0.05$). However, the urine sample obtained by our newly developed centrifuged method was cleaner, lucid and homogeneous. This preliminary study shows that the centrifugation method, although time consuming, can be reliable as it does not have statistically different results from routine practice. Long-term use of the centrifuge method may potentially reduce device maintenance, repair and consumption costs. According to these initial findings, positive effects of using centrifuge method for a long time on column costs and replacement processes can be expected in future studies © 2022 NTMS.

Keyword: LC-MS/MS; Urine; Toxicology; Morphine; Screening; Validation.

1. Introduction

Although mass spectrometry (MS) technique has high selectivity, other molecules included in the same urine matrix is very difficult to separate. The MS technique is a system designed to separate the substances depending on the m/z (mass/charge) ratios. MS, when combined with the separating liquid chromatography (LC) system (LC-MS), have unique features as both systems are combined Tandem Mass Spectrometry

Cite this article as: Eren E, Göncü Sürü A, Yılmaz N and Oncu C. Strategy for successful urine sample preparation for LC-MS/MS device at drug verification laboratory. *New Trend Med Sci* **2022**; 3(2): 91-97. Doi: 10.56766/ntms.823790

(LC-MS/MS) system has been designed and started to be used in routine practice (1, 2).

LC-MS/MS system has been frequently preferred in many advanced hospital laboratories on recent times due to its superior features such as sensitivity, speed and selectivity for detection and identification of toxic/non-toxic molecules in urine. Undoubtedly, urine sample preparation for LC-MS/MS is very important because the toxic or non-toxic analytes that are intended to be measured must be accurately targeted and they must be of the appropriate amount. Huge matrix effects in urine result in ion suppression (loss of signal) or ion enhancement (gain in signal). Also, matrix effects have a negative impact on the accuracy, precision, and robustness of the method (2, 3). As well as, a good illicit drugs verification method needs to be selective, accurate, sensitive, easy to use and automated.

However, the routine urine sample preparation techniques that are widely used today may not be appropriate for LC-MS/MS because, there are only a few simple urine sample preparation methods described in literature for urine screening with LC-MS/MS (4). Therefore, the most difficult and time-consuming step is the routine urine sample preparation phase. When routine minimal sample preparation procedures are combined with short analysis times, large amounts of endogenous species can potentially coexist with the target analyte in urine. To date, limited reports on routine urine sample preparation for LC-MS/MS have been published (1-4).

Moreover, when clear and good samples are not used, these shorten the column life, increases device downtime times, and the costs of manpower and equipment maintenance. Today, when preparing urine sample for drug analysis with LC-MS/MS, direct dilution of the urine sample or expensive extraction procedures are applied (1-4).

For example, some of the alternative methods for routine urine sample preparation are complex procedures such as; urine dilution with different solvents, protein precipitation and filtration with several precipitating agents, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) respectively. Of course, concentrating the urine analyte helps to increase sensitivity and thus reach lower detection limits (5, 6). Moreover, these complex concentering procedures removes the interfering strong matrix elements which alter the peak measurement or elute together with the target analyte (phospholipids, salts, proteins, nucleic acids, sugars, etc.) (4). However, there is not a complete consensus on the preparation of concentrated urine in the validation laboratory in previously published articles. Liquid-liquid extraction has a long history and, although other techniques are available, this technique is still accepted. More recently, liquid-solid extraction or, as it is more often called, SPE, has gained more importance. As expected, the role of routine urine sample preparation is to remove interferences from sample matrix and improve analytical system performance for LC-MS/MS (5-6). However, as mentioned above, there is no study in the literature demonstrating the effect of precipitation by centrifugation prior to injecting urine sample.

Therefore, in this study, it is aimed to determine whether the interfering substances removed from the urine by the long-term centrifuge procedure in the routine urine preparation in the validation laboratory have an effect on the results of the illegal drug use measured in the LC-MS/MS. Firstly, we aimed to reduce the effect of urine matrix with this method. We hypothesized that the results would be more cost-effective and reliable, even if the laboratory workload and analysis time increased as expected.

2. Material and Methods

2.1. Subjects and Sample Preparation

The routine administration samples of negative 50 patients who were sent to our laboratory to determine the use of illegal drugs were used to compare two different sample preparation methods (A and B). The samples were stored for maximum 30 days (-80 °C) and then analyzed. Illicit drug analytes were prepared with low and high concentrations of standards and studied with method A and B. High and low concentration illicit drug standards added equal amounts (1ppm) to the urine and the matrix effect of urine samples was determined.

The routine illicit drug tests were added in urine samples; 6 Acetyl Morphine Hydrochloride, MDMA, MDEA, MDA, Benzoylecgonine, Codein Hydrochloride, DL-Amphetamine free base, DL-Metamphetamine free base, Lorazepam, Nordiazepam, Oxazepam, Temazepam, Morphine Monohydrate, Dihydrocodeine Hydrogen. The relevant expert laboratory personnel were responsible for the performance of this analysis. The study was conducted in accordance with the declaration of Helsinki.

Routine LC-MS/MS procedures include four main steps: sample preparation, chromatographic separation, MS detection and data ratings. For the preparation of routine urine samples in LC-MS/MS measurement in our laboratory, the sample preparation method, which was generally accepted and which was explained in the user manual of the device was preferred. In this study, the samples were divided into two parts. The first part was measured using the routine administration preparation procedure with urine-A method (Figure 1). Sample A was then injected directly into the LC-MS/MS device as shown in Figure 1. In method B, long- time centrifugation (14000 g,10 minutes) was performed on the routine urine samples. The samples were taken from the upper part of the clean urine sample and the procedures in Figure 1were performed. The B method results were compared with method A

administration samples. As a result, the results obtained in both methods were carefully recorded and analyzed.

2.2. LC-MS / MS Methods

We used the Thermo Scientific LC-MS/MS to identify illegal drug use in the verification laboratory. The device was verified according to the thermo instructions and the original column and other materials were used. LC-MS/MS analysis, Thermo Scientific Dionex Ultimate 3000 pump and Ultimate Open automatic sampler, Thermo Scientific TSQ ENDURA is done by three-stage four-pole mass spectrometer. Thermo Scientific Hypersil Gold analytical column was used at ambient temperature. The measurement parameters and the LC-MS/MS procedure were as explained in LC Conditions; Thermo Scientific Hypersil Gold Column which is used (50×2.1 mm ×1.9 µm particle size). The auto sample receiver temperature was set to 15 °C, the column was set to +40°C in oven. The autosampler needle was rinsed before and after sample injection to avoid carry over. The mobile phase consists of 2 mM ammonium acetate and 0.2 % formic acid, 250 mL water with and 2 mM ammonium acetate and 0.2 % formic acid with 250 mL methanol.

HPLC Conditions; the same column and two mobile phase combinations were used in all samples (Table 1 and 2). LC gradient and mobile phase transitions are shown in Figure 2.

MS /MS Terms; the mass spectrometer was operated with heated electrospray ionization in both positive and negative ionization modes (HESI-II). For MS, all the conditions are shown in Figure 2.

2.3. Statistical analysis

Urines analyzed for illicit substances were measured twice using two different methods. Obtained test results were evaluated by statistical analysis. The statistical analysis was performed using MedCalc© Statistical Software version 15.8 (MedCalc Software® bvba, Ostend, Belgium; https://www.medcalc.org; 2018). The Kolmogorov-Smirnov for normal distribution and paired sample test was used to assess the distribution of constant variables. A P-value of <0.05 was considered statistically significant.

3. Results

Screening of a wide range of compounds from various matrices, such as urine, is challenging, but LC-MS/MS has proven to be suitable for such applications. Briefly both urine preparation methods (A and B) used in this study met our analytical standard criteria. Therefore, no significant statistical difference was found between sample A and Sample B preparation among the illicit substance measurements in urine (Table 3, p>0.05). In both methods the sensitivity and linear dynamic ranges were may be appropriate for clinical use to monitor drug use in urine. However, the duration of the urine preparation was 20 minutes longer in the sample B compared to sample A, as expected. In both standard sample preparation methods (sample A, sample B), the accuracy and dilution integrity of the methods were acceptable for the quantitative urine drug tests (Figure 3and 4).

Moreover, the analytes were stable under the conditions specified in the stored samples and did not show a significant difference over a month. The measurements of both samples stored for one month were not different in all parameters compared to fresh urine results. Analytes were stable during sample preparation and storage under the stated conditions (data not show). The centrifugation of the samples allowed to obtain a clearer urine sample, but the analysis time was longer for at least 20 minutes in method B.

Table 1: Chemicals used for analysis, certified and unmarked certified standards and brand and origin of the column used.

Chemical	Brand	Country
Acetonitrile	Carlo Erba	France
Propanol	Carlo Erba	France
Ammonium Acetate	Carlo Erba	Germany
Formic Acid	Carlo Erba	Germany
Methanol	Carlo Erba	France
Beta-glucronidase enzyme	Covachem	Germany
Internal Standard (CRM-marked)	Chiron	Norvey
Internal Standard (CRM-un-marked)	Chiron	Norvey

ISTD: Marked Standard.

Table 2: Certified and unmarked reference materials used during analysis.

Labeled Internal Standards (1ppm)	Standard (1ppm)
DL-Amphetamine-d5 Hydrochloride	DL-Amphetamine
Morphine-d3 Hydrochloride	Morphine
Benzoylecgonine-d3	Benzoylecgonine
MDA-d5 Hydrochloride	MDA
Lorazepam-13C6(7-chlorobenzo- 13C6-d5)	Lorazepam
Nordiazepam-d5(phenyl-d5)	Nordiazepam
Oxazepam-d5(phenyl-d5)	Oxazepam
Temazepam-d5(phenyl-d5)	Temazepam
Codeine-N-Methyl-d3 Hydrochloride	Codeine
Methamphetamine-d5 HCl	Methamphetamine
MDMA-d5 HCl	MDMA
(+,-) -MDEA-D5 Hydrochloride(Ethyl d5)	(+,-) –MDEA
6-Acetylmorphine-d3 HCl	6-Acetylmorphine
(-) - Trans-delta 9 - THC-d3 (pentyl-5,5,5-d3)	(+-) - Trans-11 Nor-9-carboxy delta 9 THC

Table 3: The results of the illicit substance measurements in sample A and Sample B.

Standard analytes added to urine 1ppb	Non-centrifuge Low Concentration	Centrifuge Low Concentration	Non-centrifuge High Concentration	Centrifuge High Concentration
DL-Amphetamine Free Base(ng/ml)	185,24±4.42	187,60±4.64	312,78±3,69	314,27±3,85
DL-Metamphetamine Free Base(ng/ml)	181,74±3.72	187,78±4.94	312,30±4.02	315,49±4.27
MDA(ng/ml)	186,25±4.38	187,72±4.86	312,93±5.03	314,75±5.72
MDMA(ng/ml)	191,10±3,85	187,69±3,05	312,33±4,65	316,71±5,57
MDEA(ng/ml)	183,78±4,76	187,87±4,23	312,59±4,69	312,09±4,36
Lorazepam(ng/ml)	153,02±2,57	156,07±2,73	249,87±3,75	234,37±3,84
Nordiazepam(ng/ml)	151,31±2,94	150,62±2,83	250,15±3,65	248,88±3,49
Oxazepam(ng/ml)	153,00±2,58	150,61±2,42	250,15±4,03	257,90±3,05
Temazepam(ng/ml)	150,45±2,63	150,89±2,01	250,22±3,14	241,02±3,35
11 nor THC-COOH(ng/ml)	11,18±0,38	11,17±0,32	18,41±0,54	18,04±0,73
Benzoylecgonine(ng/ml)	76,98±1,35	75,07±1,43	126,38±1,74	125,06±1,52
6 Acetyl Morphine Hydrochloride(ng/ml)	7,86±0,12	7,69±0,51	12,39±0,78	12,60±0,82
Codeine Hydrochloride(ng/ml)	227,08±1,42	224,97±1,48	373,83±0,39	374,39±0,48
Morphine Monohydrate(ng/ml)	226,21±0,48	225,64±0,51	366,47±1,12	379,31±10

* Kolmogorov-Smirnov: all parameters accepted normal distribution, Mean± standart deviation(SD), Paired sample test p>0.05 for all paraters.

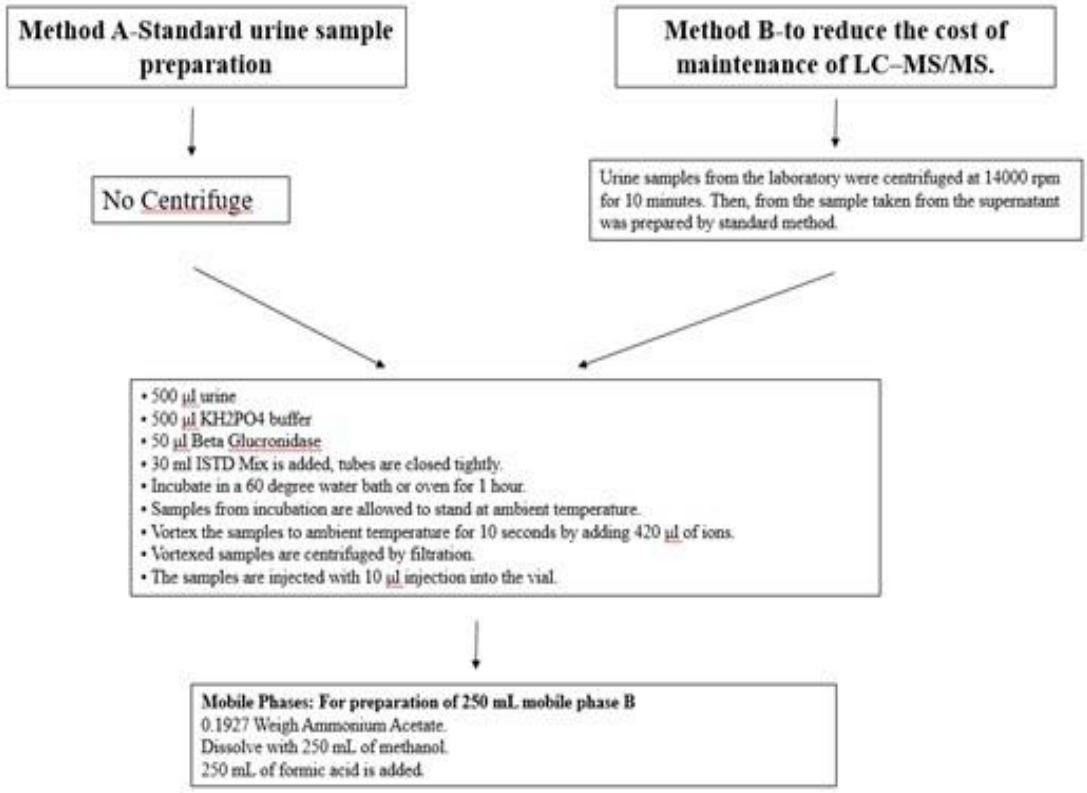

Figure 1: : Preparation of Samples.

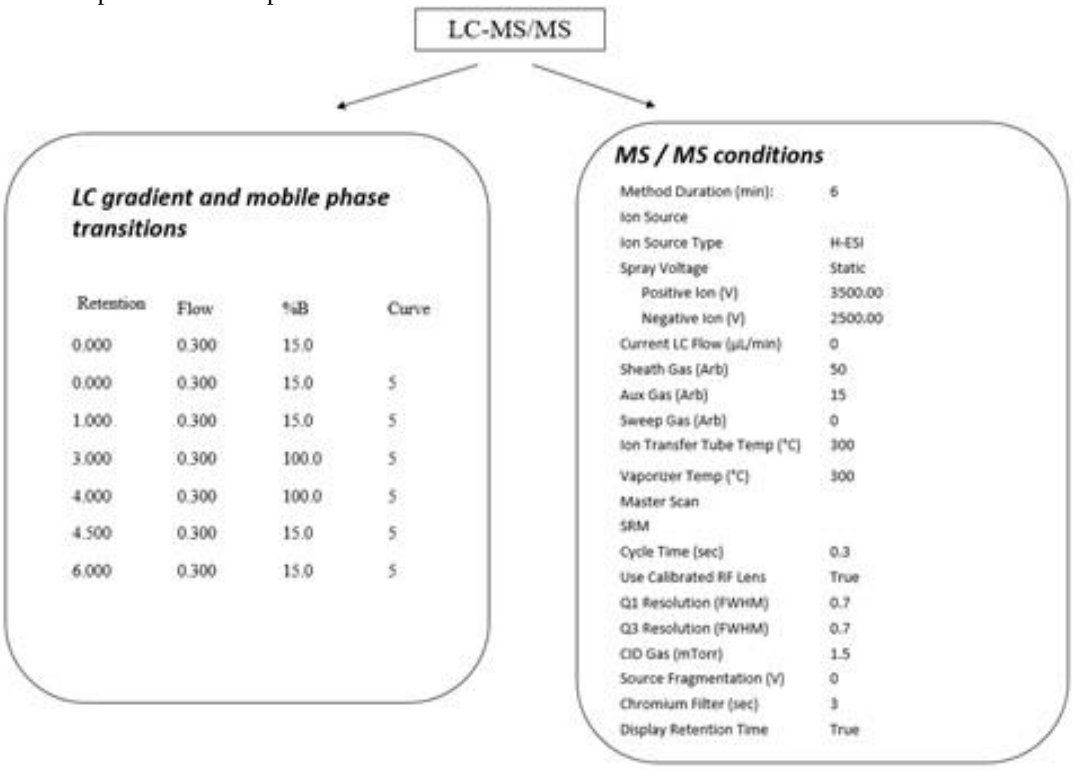

Figure 2: LC gradient and mobile phase transitions and MS/MS conditions.

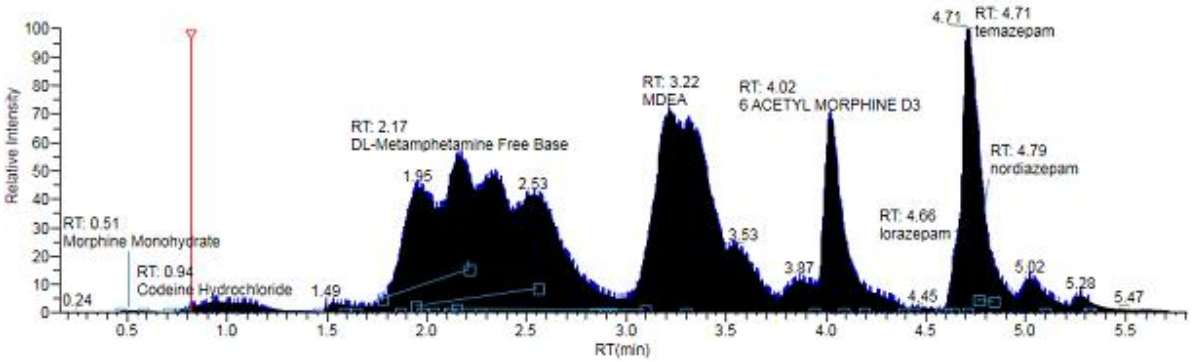

Figure 3: Method A example chromatogram.

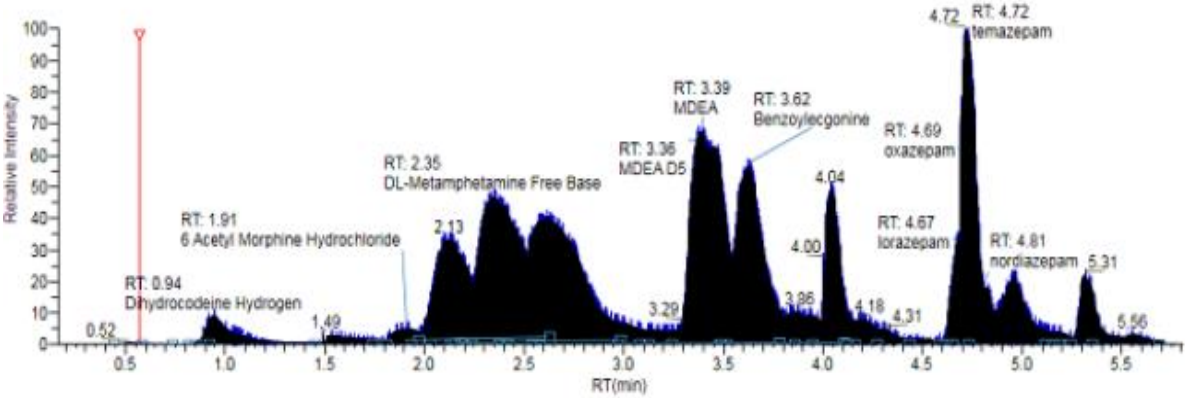

Figure 4: *Method B example chromatogram*.

4. Discussion

Our results show that urine pre-centrifuges have no statistical effect on the test results obtained. In laboratories, it is important to provide timely and rapid results, but another important feature is to reduce the equipment maintenance costs. We could not determine whether the equipment maintenance costs decreased, because the urine preparation method B was not used for a long time in our laboratory. Regarding this preliminary study, we can conclude that the long centrifugation procedure for urine (Method-B) used in our validation laboratory can reduce the urinary matrix effect when compared to the initial findings. Moreover, more clear urine samples did not change the results of toxicological analysis. Unfortunately, the commonly used complex urine preparation methods cause challenges in practice in terms of filtration and chemical separation methods and these are expensive (7-9).

Routine immunological tests are widely used to screen illicit drug use. In case of a positive result, sometimes an additional selective verification analysis may be required. Immunoassay tests are simple and fast, but not precise. Furthermore, immunological screening assays are not very selective. In addition to the compounds in a group, other structural compounds may result in an incorrect positive test due to cross-reactivity (4, 10).

We could not obtain data for long-term use of this new method for in terms of device maintenance, repair and consumption costs. However, it can be determined in more comprehensive studies can be conducted to see whether the LC-MS/MS device will reduce the maintenance costs or not (4, 8-11). In this study, we showed that a simple but time-consuming urine preparation method for the first time in urine preparation did not influence the results. Our hypothesis is that the cost of replacing the very expensive parts as columns and LC-MS/MS device will be reduced by the use of clean and urine samples. However, the initial findings suggest that column costs and replacement processes can be positively affected. In this way, we may confirm this hypothesis in the future by using the method-centrifuge in routine urine analysis for a certain period of time in our routine practices (12). However, the urine sample obtained by our newly developed method is clean, clear and homogeneous. The results of these preliminary studies indicate that although the new method is time-consuming, it can be reliable.

5. Conclusions

Consequently, this study is a pilot study and we can say that long-term pre-centrifugation does not have a negative effect on routine LC-MS/MS toxicological test measurements.

Limitations of the Study

This study is on the preliminary and includes the development and comparison of purely technical methods. The effectiveness of different urine purification techniques should be supported by clinical studies.

Acknowledgement

Thank you to the laboratory technicians of toxicology LC-MS/MS

Conflict of Interests

None of the authors have a conflict of interest.

Financial Support

There is none finansal support.

Author Contributions

Study conception and design: E.E; Data collection: E.E, A.G; Analysis and interpretation of results: N.Y, E.E; Draft manuscript preparation: N.Y. C.O. All authors reviewed the results and approved the final version of the manuscript.

Ethical Approval

A Methodical Tecnical study, no ethics committee approval was received for this study from the ethics committee of Antalya Training and Research Hospital.

Data sharing statement

All data relevant to the study are included in the article.

Consent to participate

Methodical Technical study patient material and informed consent were not obtained.

Informed Consent

A Methodical Technical study patient material and informed consent were not obtained.

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