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Stability of (1R,2S)-(-)-Ephedrine hydrochloride in *Candida albicans*-inoculated urine and blood samples

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ABSTRACT

The stability of drugs in biological evidence during collection and storage is of particular concern in forensic investigations. Microbes in the samples and other elements are an essential component of these investigations. In the current study, the HPLC method was used to examine the stability of (1R, 2S)-(-)-Ephedrine hydrochloride in plasma and urine samples inoculated with *C. albicans* after storage at 37 °C for 48 h and −20 °C for six months. In the stability experiment, $MIC_{50\%}$ of (1R, 2S)-(-)-Ephedrine hydrochloride was applied according to MIC and MFC that were determined in this work. This drug had MIC and MFC of 50 and 100 ppm, respectively. In HPLC analysis, the standard (1R, 2S)-(-)-Ephedrine hydrochloride had a retention time of 1.63 and was used to identify this drug in samples that had or had not been exposed to *C. albicans*. The findings demonstrated that within 48 h at 37 °C, *C. albicans* had an impact on the drug concentration (10% and more than 15%, respectively, were lost in plasma and urine samples inoculated with *C. albicans*). In plasma samples, the drug content remained stable at −20 °C for three months, although it degraded in urine samples after one month. In plasma and urine samples, the concentration reduction had surpassed 70% and 50% by the sixth month, respectively. The results of this investigation show that *C. albicans* can change the stability of (1R, 2S)-(-)-Ephedrine hydrochloride in plasma and urine samples that contain $MIC_{50\%}$ of Ephedrine hydrochloride.

1. Introduction

According to one definition, "microbial forensics" is "a scientific discipline dedicated to assessing evidence from a bioterrorism activity, a biocrime, or an unintended release of microbial pathogens or their toxins for the sake of attribution." (Budowle et al., 2003). This science studies microbes as spatial and temporal physical evidence, as well as the direct and indirect effects of microbes on biological samples collected from crime scenes or from drug users (Burcham and Jordan, 2017). One of the most important issues discussed in a lot of research is the persistence and stability of drug concentrations in biological samples. These studies confirmed that microbes have a direct impact on the concentration of drugs in blood, urine, and other biological samples (Djilali et al., 2022). Ephedrine, an alkaloid derived from the plant *Ephedra vulgaris*, is the basis for stimulant drugs like amphetamine. In the past, these drugs were primarily used for medical treatments, but their use has been significantly reduced since the 1970 s, when the high risk of addiction and abuse was recognized (Morelli and Tognotti 2021).

The detection of ephedrine in biological samples and the estimation of its concentration are critical for many medical issues or the use of this drug in areas other than medicine, such as sports and bodybuilding. For this purpose, gas chromatography/mass spectrometry (GS/MS), high-performance liquid chromatography (HPLC), and spectrophotometry methods have been used. (Behbahani et al., 2019, Dindar et al., 2020, Eskandrani 2022). The pathogenic *Candida* strains (such as *C. albicans*) spread through bodily fluids and causes a range of diseases (including candidiasis, candidemia, and urinary tract infections). It has been identified in blood and urine samples, implying that it may be present in biolog-

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ical samples collected from candidiasis patients (Odabasi and Mert 2020). A microbial load, which can be from external or original contamination in the biological samples, such as from people with a microbial infection, influences the stability of ephedrine and its derivatives in biological samples. The study's hypothesis is that yeast in a person's plasma and urine can alter the stability of ephedrine in biological samples used in forensic examinations. This could result in incorrect chemical analysis findings. The purpose of this research was to determine the stability of ephedrine in plasma and urine samples inoculated with *C. albicans*.

2. Methodology

2.1. Candida albicans strain

In this investigation, *Candida albicans* (Robin) Berkhout 60193™ was used. The macroscopic characteristics of this strain include that, after three days of incubation on potato dextrose agar, its colonies become cream-colored and dull, and its mycelial border frequently appears wrinkled. These yeast cells are hyaline, spherical (globose) to short-ovoid in size (approximately 2–7 × 3–8.5 um), singular cells or buds, and rarely form short chains. This yeast is classified as follows: eukaryotic cells (domain), fungi (kingdom), Ascomycota (phylum), Saccharomycetes (class), Saccharomycetales (order), Debaryomycetaceae (family), *Candida* (genus), and *albicans* (species) (Belvoncikova et al., 2022).

2.2. Chemicals and media

(1R, 2S)-(-)-Ephedrine hydrochloride was purchased from Cerilliant Corporation, USA (a company that specializes in the production of reference chemical compounds for the purpose of toxicology and forensic analysis). Mueller-Hinton broth (MHB), Mueller-Hinton agar (MHA), Potato dextrose broth (PDB), Potato dextrose agar (PDA), Nutrient broth (NB), Nutrient agar (NA), and Blood agar base (BA) were purchased from Oxoid Ltd, Basingstoke, UK. All media were prepared according to the manufacturer's instructions.

2.3. Urine and plasma samples

Drug-free plasma and drug-free urine samples were obtained from the Bio House Medical Laboratory (BHML), Riyadh, Saudi Arabia. It was confirmed that the plasma and urine are microbial contamination-free samples using the cultivation method in different media, and incubation aerobically and anaerobically at different temperatures.

2.4. Effect of ephedrine derivatives on C. albicans

The inhibiting and cidal effects of (1R,2S)-(-)-Ephedrine hydrochloride were investigated using a two-fold microdilution assay for determining minimal inhibitory concentrations (MICs) and minimal fungicidal concentrations (MFCs). Briefly, aqueous solutions of the drug (100 ppm) were prepared and *C. albicans* was cultivated on PDA at 35 ± 1 °C for 20 h. The microbial suspension was prepared from single colonies to obtain 0.6 optical density (OD) at 570 nm. NB were prepared, and distributed in 96 well-microplates (each well received 90 µL). After that, all wells received 10 µL of microbial suspension except control wells that received 10 µL of sterile normal saline (0.89% sodium chloride). 100 µL of the drug solution was added into the first well, and after a complete mixture, the second well received 100 µL from the first well, the process continued to the last well in the same row, and from that well,100 µL were transferred out of the plate (discarded in 70% ethanol solution). The incubation was aerobically done at 35 ± 1 °C for 24 h then the turbidity was recorded for all wells. MFCs were determined using the subcultivation method according to (Sukhikh et al., 2022) for calculating the lowest concentration that had the ability to kill all microbial cells.

2.5. Stability experiments

The stability of (1R, 2S)-(-)-Ephedrine hydrochloride in urine and plasma samples inoculated with *C. albicans* was studied as follow:

Treated groups: In these groups, $MICs_{50\%}$ of drug were added to urine and plasma samples inoculated with *C. albicans* ($5x10^6$ yeast cell/mL).

Control groups: Control groups: Four control groups were applied at this stage. The first group was urine and plasma samples without drugs or microbes (the "drug-free and sterile groups"); the second group included urine and plasma samples only treated with drugs (the "sterile groups"); the third group was urine and plasma inoculated with *C. albicans* (the "non-sterile groups").

All the above groups were preserved at −20 °C for 6 months and at 37 °C for 24 h and 48 h. During that period, samples were collected at 24 h, 48 h, one, two, three, four, five, and six months for chemical analysis. The stabilization of the drugs in urine and samples was performed according to Jimenez et al., (2006) with some modifications regarding the preparation of samples and the extraction. All storage and experiment conditions were under constant surveillance.

The quantification and calibration using serial concentrations (0.3, 0.7, 1.5, 3.1, 6.2, 12.5, 25, 50, 100 µg/ml) of (1R, 2S)-(-)-Ephedrine hydrochloride were carried out using HPLC analysis. The samples were analyzed using HPLC equipment (Agilent 1290, HPLC, CA, USA) that contains a pump, an ultraviolet detector, and an automatic sample injector. The (1R,2S)-(-)-Ephedrine hydrochloride was extracted from the samples prior to injection by adding 1 mL of methanol to the sample (100 µL) and incubating it for 24 h in a shaker at 225 rpm. The alcoholic layer was removed, and the extraction from the remaining solution using methanol was repeated three times, with the alcoholic layer being removed and all alcoholic layers being brought together. A rotary vacuum was used to remove 90% of the solvent (methanol) during the concentration.

The column (type, length, and temperature), mobile phase (solvent, flow speed), injected size, and estimation condition (type and wavelength) were listed in Table 3.

2.6. Experimental design and statistical analysis

A completely randomized design is used for one factor, where time is the block according to the following mathematical model:

$$Y_{ij} = \mu + T_i + \beta_j + \xi_{ij}$$

Where,

Y = the observation of experiment unit that treated with (1R, 2S)-(-)-Ephedrine hydrochloride and microbes (i) at time (j).

μ = mean of (1R, 2S)-(-)-Ephedrine hydrochloride.

T = effect of treatment (i).

B = effect of time (j).

ξ = random error.

OriginPro 2018 was used for statistical analysis of variance, and the results were expressed as means ± standard error.

3. Results

Antimicrobial testing of (1R, 2S)-(-)-Ephedrine hydrochloride revealed that the compound has biological activity against *C. albicans*. The MIC and MFC concentrations were 50 and 100 ppm, respectively (Fig. 1). The results showed that the standard deviations were zero because the three replicates (N = 3) of the tests had the same value for MIC and MFC. The stability test was designed based on the MIC and MFC results, with $MIC_{50\%}$ (25 ppm) chosen to assess the effect of *C. albicans* on the stability of (1R, 2S)-(-)-ephedrine hydrochloride in plasma and urine samples.

The retention time (min.), height (mAU), and area (%) of (1R, 2S)-(-)-Ephedrine hydrochloride determined in the plasms sample without *C. albicans* inoculation are shown in Fig. 2. The standard curve was made by dissolving 200 ppm of the compound references in 1 mL of methanol, and the compound was detected in plasma using HPLC analysis under the conditions and column type shown in Table 1.

Table 2 summarizes the stability of (1R,2S)-(-)-Ephedrine hydrochloride in plasma inoculated with *C. albicans* over various storage periods at −20 °C. The HPLC analysis was performed at 214.4 nm under the same conditions described in Table 1. The data in Table 2 show that after 48 h of storage at 37 °C, the concentration of (1R,2S)-(-)-Ephedrine hydrochloride in plasma samples inoculated with *C. albicans* dropped by 10%, while it took six months to drop by 12.28% when stored at −20 °C. When stored at −20 °C for six months, the concentration in the samples inoculated with *C. albicans* decreased by more than 70%, while the compound retained its full concentration in the control sample that was not treated with *C. albicans*. Fig. 3 shows that the concentration of (1R,2S)-(-)-Ephedrine hydrochloride decreases significantly (p less than 0.05) in plasma samples inoculated with *C. albicans* when stored at 37 °C for 48 h, as well as at −20 °C for four, five, and six months.

Fig. 4 illustrates the retention time (min.), height (mAU), and area (%) of (1R,2S)-(-)-Ephedrine hydrochloride in sterile urine samples. Table 3 shows the effect of *C. albicans* on the concentration of (1R,2S)-(-)-Ephedrine hydrochloride in urine samples stored at 37 and −20 °C. Within 48 h, more than 15% of the (1R,2S)-(-)-Ephedrine hydrochloride concentration was lost during preservation at 37 °C. In contrast to what happened with plasma samples, the concentration of the compound began to fall in the first month of storage at −20 °C and continued to fall until it was less than 50% in the sixth month. The compound concentration decreased significantly in all urine samples compared to the control group, with the exception of samples stored at 37 °C for 24 h, where the concentration was 100% (Fig. 5).

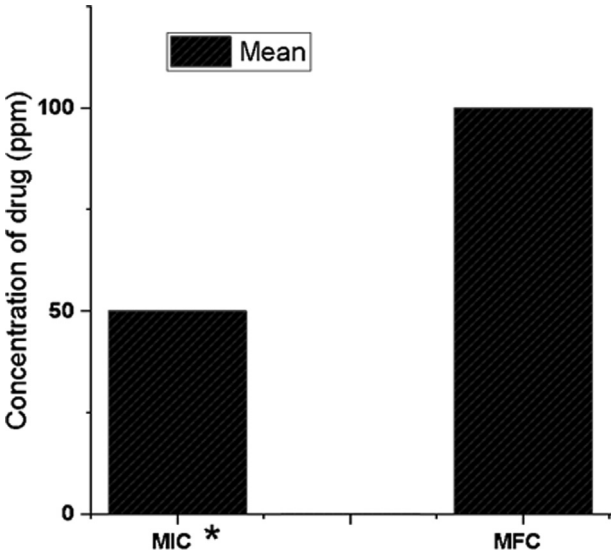

Fig. 1. Minimal inhibitory concentrations (MICs) and minimal fungicidal concentrations (MFCs) of (1R,2S)-(-)-Ephedrine hydrochloride. *The tests were replicated three times (N = 3), and standard errors for all treatments were zero for all treatments.

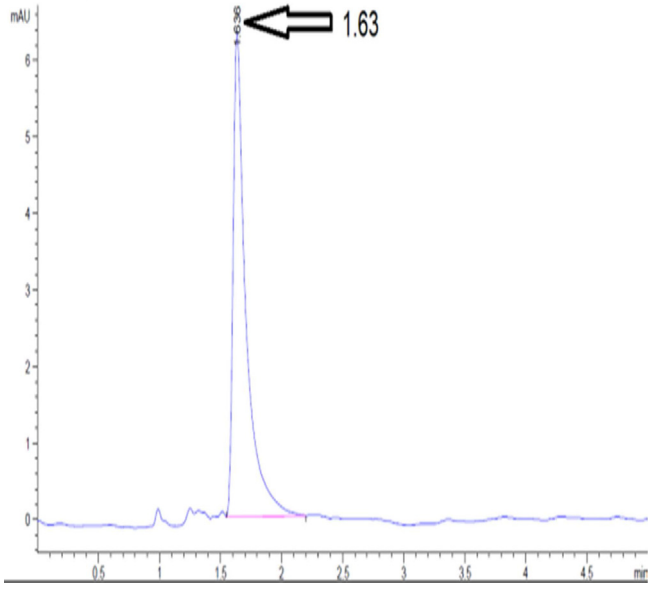

Fig. 2. HPLC examination of sterile plasma samples treated with (1R, 2S)-(-)-Ephedrine hydrochloride. The retention time corresponded to the standard curve results of (1R, 2S)-(-)-ephedrine hydrochloride.

Table 1
The operating conditions and column type used in HPLC analysis.

Column	
Type	SB-C18(Agilent)
length	150 mm
temperature	23 °C
Mobile phase	
Solvent	Acetonitrile/Methanol(20:80%)
Flow speed	1.0 mL/min
Injector	
Type	Automatic
Injected size	1.0 μL
Estimation conditions	
Estimation type	ultraviolet detector
Wave length	214 nm

4. Discussion

One of the most important scientific questions that many scientists and researchers have investigated is how drugs remain stable in biological samples stored for short and long times. This sentence should be edited to "There is a possibility that the samples are contaminated with microbes or were collected from infected patients, which could jeopardize the results of future tests and the overall outcome of the criminal case. Contamination may occur after the sample has been collected. Another possibility is that it occurs prior to sample collection, assuming that the pathogenic microbe is infected and the sample was collected while it was carrying the microbe. *C. albicans* strains are frequently detected in clinical blood and urine samples (El-Baz et al., 2021), and their presence

Table 2
Stability of (1R, 2S)-(-)-Ephedrine hydrochloride (EH) in plasma inoculated with *C. albicans* during different storage periods at −20 °C. (HPLC analysis at 214.4 nm).

Treatment	Retention time (min.)	Height (mAU) and area%	Comment
Standard curve EH	1.64	8.9924 (100%)	Major one peak
Plasma (Control) without bacteria or EH	1.21	66.831(88.48%)	Major one peak. Some compounds were found at
	1.56		different times and with an area not exceeding 8%.
	2.85		
Plasma with EH	1.63	6.3574 (100%)	One peak
Plasma with *C.albicans* and without EH	1.26	588.670 (100%)	One peak
Plasma with *C. albicans* and EH after Zero time	1.632	6.84616 (100%)	One peak
Plasma with *C. albicans* and EH after 24 h, at 37 °C	1.636	6.30785 (100%)	One peak
Plasma with *C. albicans* and EH after 48 h, at 37 °C	1.61	57.123 (90%)	Major one peak. Some compounds were found at different times and with an area not exceeding 4%.
Plasma with *C. albicans* and EH after one month at −20 °C	1.63	6.2850 (100%)	One peak
- after two months at −20 °C	1.63	6.39346 (100%)	One peak
- after Three months at −20 °C	1.63	6.18727 (100%)	One peak
- after four months at −20 °C	1.63	9.701 (87.72%)	Major one peak. Some compounds were found at
	1.25		different times and with an area of 12.27%.
	1.037		
- after five months at −20 °C	1.64	6.079 (86%)	Major one peak. Some compounds were found at different times and with an area not exceeding 5%.
- after six months at −20 °C	1.63	6.43736(29.26%)	Six peaks
	2.41	9.78844(28.36%)	
	1.25	4.77332(14.41%)	
	1.02	7.77890(14.09%)	
	1.37	3.37338(9.61%)	
	1.10	2.17291(4.23%)	

may have an impact on the examination results if drugs were used during the infection. In this study, we attempted to test this hypothesis by introducing *C. albicans* into blood and urine samples containing the drug. "Does *C. albicans* have the ability to affect the stability of the drug ((1R, 2S)-(-)-Ephedrine hydrochloride) in blood and urine samples stored at 37 and −20 °C?" was the question. One major limitation of our current study is that the previous hypothesis was tested in the laboratory, and the effect of yeast infection on drug concentration in blood or urine necessarily requires the use of experimental animals. But another goal of the laboratory study is to find out what happens to the drug when yeast is present in blood and urine samples for short and long periods of time.

(1R,2S)-(-)-Ephedrine hydrochloride inhibited and killed *C. albicans* at concentrations of 50 and 100 ppm, respectively. This is not the first study to demonstrate that ephedrines can inhibit pathogenic bacteria and yeast. This sentence should be edited to "Fazeli-Nasab and Mousavi (2019) demonstrated that *Pseudomonas aeruginosa* strains isolated from clinical samples can be inhibited by some of this drug's derivatives. This finding indicates that, in addition to the disastrous psychological effects caused by this drug and its derivatives, there may be a negative effect on the natural microbial flora in the human body, because the yeast tested in this study is considered a natural microbial flora in many sites in the human body, including the mouth and digestive tract, and is also an opportunistic pathogenic yeast (d'Enfert et al., 2021, Talapko et al., 2021).

The preservation and maintenance of biological samples used as forensic evidence necessitates extreme caution, as any change in this evidence can cause a significant disruption in the course of any criminal case. The scientific reports have demonstrated the

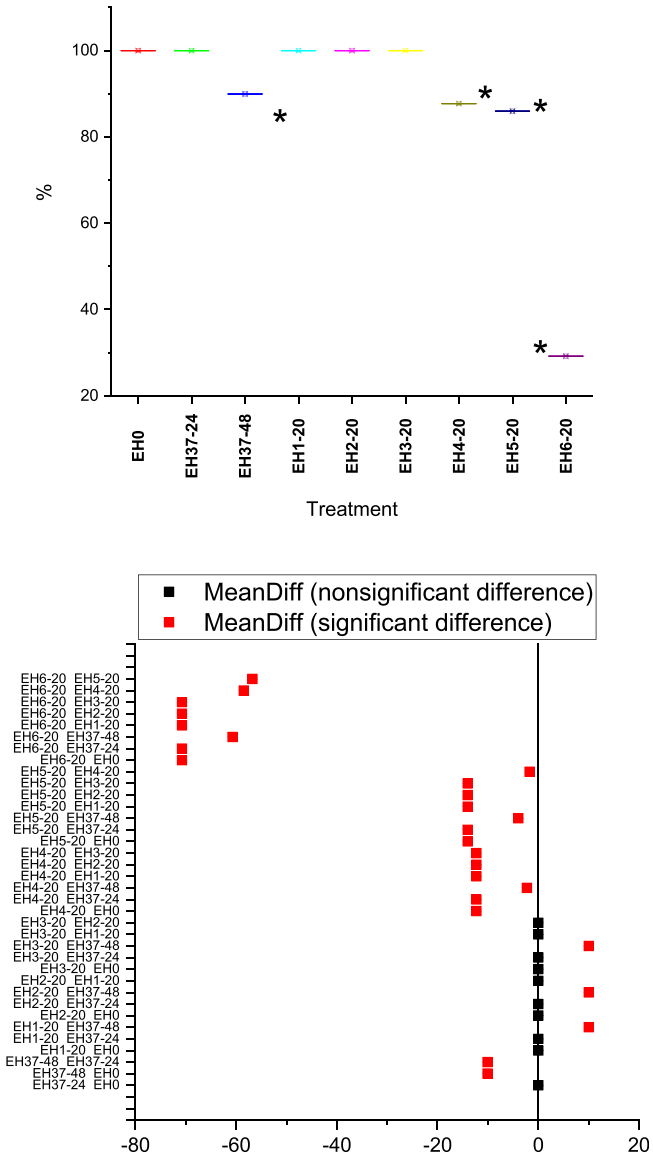

Fig. 3. The effect of storage time at 37 and −20 °C on the stability of (1R, 2S)-(-)-Ephedrine hydrochloride (EH) (%) in *C. albicans*-inoculated plasma. (N = 2) * Significant differences exist at the 0.05 level when compared to the EH group. EH = Plasma with EH, EH0 = Plasma with *C. albicans* and EH after Zero time, EH37-24 = Plasma with *C. albicans* and EH after 24 h, at 37 °C, EH37-48 = Plasma with *C. albicans* and EH after 48 h, at 37 °C. EH1-20, EH2-20, EH3-20, EH4-20, EH5-20, and EH6-20 = Plasma with *C. albicans* and EH after one, two, three, four, five and six months at −20 °C respectively.

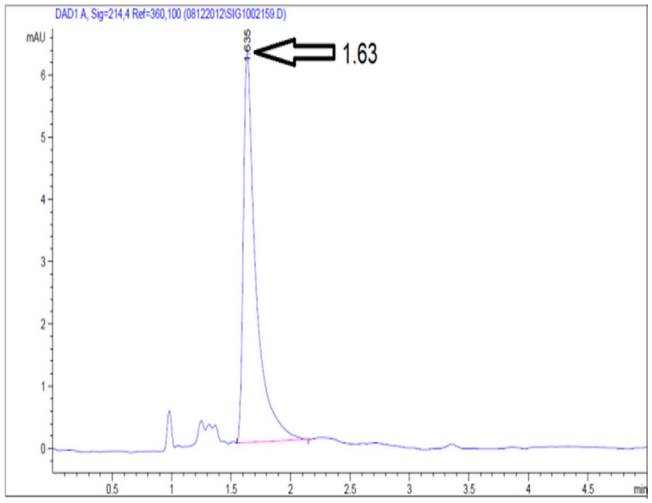

Fig. 4. HPLC examination of sterile urine samples treated with (1R, 2S)-(-)-Ephedrine hydrochloride The retention time corresponded to the standard curve results of (1R, 2S)-(-)-ephedrine hydrochloride.

impact of microbial contamination on biological samples stored for short or long periods of time. Skopp stated that the stability of these compounds in biological samples is a critical requirement in drug analysis. He also confirmed that instability could occur as a result of a variety of enzymatic reactions or chemical actions (Skopp 2022). The microbial degradation of the drug in biological samples after collection, whether the sample was a carrier of the microbe or due to external contamination, causes a change in the analysis results, which can be significant. Morphine glucuronides, for example, are converted to free morphine in blood samples, and enteric microorganisms can biomodify nitrobenzenes (Carroll et al., 2000, Kerrigan 2013). Our findings showed that *C. albicans* can reduce the stability of (1R,2S)-(-)-Ephedrine hydrochloride in plasma and urine samples within 48 h, as well as change the stability of this drug during storage at −20 °C. Ephedrine hydrochloride ($C_{10}H_{16}C_lNO$) is an alkylbenzene that can stimulate the central nervous system, according to PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/Ephedrine-hydrochloride). Its chemical and physical properties indicate that it can donate three hydrogen atoms while gaining two. Its pharmacology and biochemistry properties include adrenergic activity, sympathomimetic activity, central nervous system stimulation, and vasoconstrictor activity.

The concentration of the (1R,2S)-(-)-Ephedrine hydrochloride in samples decreased when yeast was added. This suggests that enzymes and other yeast-produced substances may have altered (1R,2S)-(-)-Ephedrine hydrochloride. The ability of (1R,2S)-(-)-Ephedrine hydrochloride to give and gain hydrogen suggests that the reactions that occurred were of the redox type, and that this was the cause of the decrease in the concentration of (1R,2S)-(-)-Ephedrine hydrochloride in plasma and urine samples containing *C. albicans*. The alkylbenzenes are known to be benzene derivatives in which one or more hydrogen atoms are replaced by alkyl groups of varying sizes, where the alkyl group is an alkane missing one hydrogen (Lambert 1993). It has been scientifically proven that a group of yeasts, such as *C. utilis*, can biotransform ephedrines via a mechanism known as microbial biotransformation. This efficiency has been used in the production of L-ephedrine, which is used to produce decongestant and antiasthmatic drugs (Rogers et al., 1997).

HPLC, a chemical analysis method, has long been recognized as a reliable method for analyzing ephedrine in biological samples. For example, extensive research has established its suitability for this purpose such as (Abdel Salam et al., 2013, AKSU DÖNMEZ et al., 2018, Kallinteris et al., 2022). The results of previous studies and our current study did not completely match due to differences in samples, extraction methods, mobile phase, and other differences. All methods, however, used the previous analysis technique for diagnosing and estimating ephedrine in biological samples based on standard drug retention times. For example, the retention time obtained in our work was 1.63, whereas it was 6.31 in Kallinteris and his colleague's work. Furthermore, when the results of previous studies were compared, these differences were discovered.

Table 3
Stability of (1R, 2S)-(-)-Ephedrine hydrochloride (EH) in urine inoculated with *C. albicans* during different storage periods at −20 °C. (HPLC analysis at 214.4 nm).

Treatment	Retention time (min.)	Height (mAU) and area%	Comment
Standard curve EH	1.64	8.9924 (98.4%)	Major one peak
Plasma (Control) without bacteria or EH	1.21 1.56 2.85	66.831 (88.48%)	One peaks. Two more peaks were found at different times and with an area not exceeding 8%.
Plasma with EH	1.63	6.3429 (100%)	One peak
Plasma with *C. albicans* and without EH	1.26	588.670 (100%)	One peak
Plasma with *C. albicans* and EH after Zero time	1.63	6.335 (100%)	One peak
Plasma with *C. albicans* and EH after 24 h, at 37 °C	1.632	6.84616 (100%)	One peak
Plasma with *C. albicans* and EH after 48 h, at 37 °C	1.635 1.020	6.38252 (84.21%) 2.18558 (15.78%)	Two peaks
Plasma with *C. albicans* and EH after one month at −20 °C	1.63 1.27	9.580 (91.41%) 6.0274 (8.58%)	Two peaks
- after two months at −20 °C	1.63 1.25	5.848 (82.39%) 8.061 (9.57%)	Two peaks. Some peaks were found at different times and with an area not exceeding 2%.
- after Three months at −20 °C	1.63 1.24	6.286 (80.69%) 1.298 (12.36%)	Two peaks. Some peaks were found at different times and with an area not exceeding 4%.
- after four months at −20 °C	1.63	6.56110(75.42%)	Major one peak and some peaks with total area less than 25%
- after five months at −20 °C	1.632	6.56110(75.42%)	Major one peak and some peaks with total area less than 25%
- after six months at −20 °C	1.638 2.41 1.01 1.25 1.37	6.25576(49.16%) 3.58291(18.37%) 3.94298(12.46%) 2.00120(11.32%) 1.69665(8.66%)	Five peaks

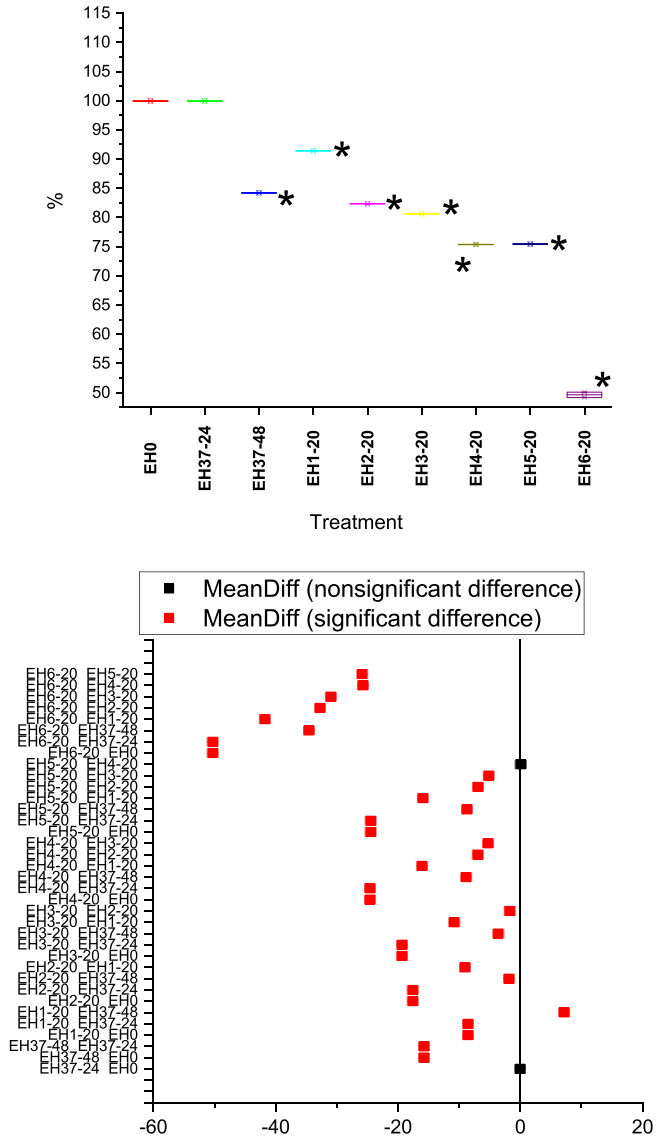

Fig. 5. The effect of storage time at 37 and −20 °C on the stability of (1R, 2S)-(-)-Ephedrine hydrochloride (EH) (%) in *C. albicans*-inoculated urine. (N = 2) * Significant differences exist at the 0.05 level when compared to the EH group. EH = urine with EH, EH0 = urine with *C. albicans* and EH after Zero time, EH37-24 = urine with *C. albicans* and EH after 24 h, at 37 °C, EH37-48 = urine with *C. albicans* and EH after 48 h, at 37 °C. EH1-20, EH2-20, EH3-20, EH4-20, EH5-20, and EH6-20 = urine with *C. albicans* and EH after one, two, three, four, five and six months at −20 °C respectively.

5. Conclusion

The findings of the present work reported that (1R, 2S)-(-)-Ephedrine hydrochloride has biological activity against *C. albicans*, MIC and MFC of this compound were 50 and 100 ppm respectively. If *C. albicans* is found in blood or urine samples containing ephedrine at concentrations lower than the inhibiting concentration, the results of the analysis are messed up and the drug concentration clearly drops in samples stored at 37 or −20 °C. This can occur due to sample contamination or when collecting samples from individuals suffering from candidiasis, a yeast infection caused by *C. albicans* strains. The results confirmed that after six months of storage at −20 °C, more than 50% and 70% of the (1R, 2S)-(-)-Ephedrine hydrochloride concentrations in plasma and urine samples, respectively, are lost when *C. albicans* is present. Further to that, when stored at 37 °C for 48 h, the drug concentrations were lost. These findings must be considered when preserving and analyzing biological samples obtained from individuals suffering from microbial infection. These findings and conclusions necessitate further investigation using appropriate laboratory and animal experiments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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