

THE IMPACT OF COLLECTION TUBE FILL VOLUME ON INTERNATIONAL NORMALIZED RATIO

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Abstract

Introduction: Pre-analytical variability currently represents the most important source of errors that can lead to inaccurate patient results in monitoring of patients being treated with oral anticoagulant therapy. The volume of blood collected is critical for accurate coagulation results. The National Committee for Clinical Laboratory Standards (NCCLS) recommends a ratio of blood to anticoagulant volume of 9:1. However, investigators have published reports which suggest that a lower ratio may be acceptable. Unfortunately the recommendations of these reports are inconsistent.

Aim: The aim of this study was to determine the impact of tube fill volume on INR values both in healthy subjects and patients receiving oral anticoagulation therapy.

Methods: INR values were obtained by processing coagulation specimens containing different volumes of whole blood. The study group included 30 patients taking oral anticoagulation therapy and 15 healthy volunteers. Respectively 2.5ml, 3 ml, 3.5 ml, 4 ml and 4.5 ml of whole blood was drawn into tubes containing a fixed volume of 3.2% (0.109M) sodium citrate.

Results: The INR values increased as total tube fill volumes decreased for both groups but this finding did not reach statistical significance in either group for the tube fill volumes studied.

Conclusion: For blood specimens collected in 3.2% citrate anticoagulant, a total tube fill volume of greater than 56% yielded reliable INR results

Introduction

Despite it being one of the earliest forms of therapeutic drug monitoring, controversies still surround optimal laboratory control of oral anticoagulant therapy (OAT).¹ Oral anticoagulation medication such as warfarin has a narrow therapeutic index. This means that there is a narrow margin between inadequate doses that can lead to recurrent thrombotic events and excessive anticoagulation which predisposes to haemorrhage. Oral anticoagulation therapy thus requires frequent international normalized ratio (INR) testing to ensure safe and effective dosing which will reduce the significant risk of complications due to excessive anticoagulation.²

Because of the greatly improved laboratory instrumentation and the availability of highly sensitive reagents, pre-analytical variability currently represents the most important source of errors that can lead to inaccurate patient results in the modern coagulation laboratory.³ These pre-analytical variables refer to any influence that, before specimen testing, causes the test result to fail to reflect the patient's *in vivo* haemostatic function.³ Up to 46% of laboratory testing errors may be attributed to the pre-analytical phase of laboratory testing with coagulation tests exceptionally susceptible to error introduced by suboptimal specimen quality.⁴ Pre-analytical variables known to affect coagulation studies can be classified into three broad categories: specimen collection, specimen transportation and storage and specimen processing. Effective management of these factors is aimed at eliminating costly, ineffective and sometimes life-threatening outcomes to patients.⁴

Variables which may influence the specimen at the time of blood collection are numerous. The choice and concentration of the anticoagulant used, the ratio of anticoagulant to whole blood and the haematocrit of the patient are but some of the factors which may cause erroneous results.

Plasma calcium is an essential requirement for many of the biochemical processes in the coagulation mechanism. The citrate in the collecting tubes used for coagulation studies inhibits clot formation by rapidly complexing with the available calcium in the plasma sample to form a soluble complex. It follows that citrate concentration is one of the major variables that can affect the result of coagulation tests.^{1, 5} The International Standards Committee on Thrombosis and Haemostasis and the National Committee for Clinical Laboratory Standards (NCCLS)⁶ have recommended guidelines to standardise whole blood collection to 3.2 g/l (0.109M) sodium citrate anticoagulant. The 3.2% sodium citrate was selected because this is the concentration used for calibration and assignment of thromboplastin International Sensitivity Index.¹ The anticoagulant should be 105 to 109 mmol/l of buffered or nonbuffered dihydrate trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$).

Previous recommendations were for the use of 3.8% (0.129M) buffered sodium citrate dihydrate. Since higher concentrations of citrate bind more calcium and also neutralise the test reagent calcium, PT measurements may be prolonged. Dilution of the plasma by citrate solution may accentuate this effect.³ As a result, INRs are significantly higher in samples collected in 3.8% citrate compared to 3.2% citrate, especially in patients receiving anticoagulant therapy.^{7, 8, 9} It is not clear what properties of the PT reagents cause them to be sensitive to the concentration of citrate anticoagulant.

Other anticoagulants e.g. oxalate, ethylenediamine tetra-acetic acid (EDTA), *etc* are not acceptable.⁶ Heparin and EDTA inhibit the coagulation process directly and also interfere with endpoint determinations.¹⁰ The labile coagulation factors (V and VIII) are unstable in oxalate which slowly forms an insoluble complex with calcium.

Chantarangkul and co-workers found that PTs for plasma collected in

Table 1: Table of mean showing INR by tube fill volume for male and female patients taking warfarin

Total Fill Volume (ml)*	Fill Volume (%)	Mean INR Males (n = 11)	Mean INR Females (n = 19)
5	100	2.67 ± 1.07	2.41 ± 0.78
4.5	89	2.80 ± 1.06	2.51 ± 0.85
4	78	2.81 ± 1.05	2.52 ± 0.85
3.5	67	2.92 ± 1.10	2.58 ± 0.90
3	56	3.10 ± 1.12	2.77 ± 1.02

*NS = not significant

Table 3: Table of Mean Showing INR by Tube Fill Volume for Healthy Volunteer Male and Female

Total Fill Volume (ml)*	Fill Volume (%)	Mean INR Males (n = 6)	Mean INR Females (n = 9)
5	100	1.09 ± 0.07	1.03 ± 0.04
4.5	89	1.10 ± 0.07	1.04 ± 0.06
4	78	1.09 ± 0.08	1.04 ± 0.06
3.5	67	1.12 ± 0.08	1.05 ± 0.05
3	56	1.16 ± 0.10	1.08 ± 0.05

*NS = not significant

Table 2: Summary of statistics of PT and INR values for subjects taking warfarin

Total Fill Volume (ml)	Fill Volume (%)	Mean ± SEM (PT) (s) (n = 30)	Median PT (s) (n = 30)	Mean ± SEM (INR) (n = 30)	Median INR (n = 30)
5	100	28.38 ± 1.71	25.77	2.5 ± 0.16	2.27
4.5	89	29.43 ± 1.83	26.98	2.6 ± 0.17	2.38
4	78	29.51 ± 1.83	26.27	2.6 ± 0.17	2.32
3.5	67	30.42 ± 1.93	27.15	2.7 ± 0.18	2.39
3	56	32.47 ± 2.09	29.20	2.9 ± 0.19	2.58

Table 4: Summary Statistics of PT and INR Values for Healthy Volunteers at Different Tube Fill Volumes

Total Fill Volume (ml)	Fill Volume (%)	Mean ± SEM (PT) (s)	Mean ± SEM (INR) (n = 15)
5	100	11.87 ± 0.70	1.05 ± 0.06
4.5	89	11.94 ± 0.76	1.06 ± 0.07
4	78	11.93 ± 0.75	1.06 ± 0.07
3.5	67	12.14 ± 0.78	1.08 ± 0.07
3	56	12.57 ± 0.89	1.12 ± 0.08

129mM citrate were longer than those collected in 105 mM both for normal and anticoagulated patients.⁵ The ratios (patient-to-normal clotting times) for the two citrate concentrations were significantly different in many instances, implying that the International Sensitivity Index (ISI) was also different. They deduced that the citrate concentration may have considerable effect on the ISI of reagents used and that the effect depended, to a large extent, on the reagent and instrument combination.

The possibility of spurious prolongation of the PT and INR due to overcitrated specimens caused by underfilling of tubes, variation of vacuum or high haematocrit is particularly worrying in the light of recommendations advocating the routine use of oral vitamin K in the management of patients on oral anticoagulant therapy with an asymptomatic increase in the INR.¹¹

Prolonged PTs result from the overcitrated of the reduced proportion of plasma in the whole blood samples of patients with raised packed cell volume (PCV) / haematocrit (Hct) levels. The implication is that the final citrate concentration in the collection tube needs to be adjusted for patients who have haematocrit values above 55%.⁶

Aim

The aim was to determine the impact of tube fill volume on INR values both in healthy subjects and patients receiving oral anticoagulation therapy.

Study Population

The study population consisted of two groups. The first group comprised 30 patients, all older than 18 years and taking warfarin. These individuals were randomly recruited from the anticoagulation clinic. Inclusion criteria dictated that: warfarin therapy should have exceeded one month, the previous INR must have been within the therapeutic range (2 to 3), the study subject's haematocrit should fall between 25% and 55% and co-existing liver disease should be absent as evidenced by the patient history and clinical records.¹²

The second group consisted of 15 healthy volunteers who were older

than 18 and randomly selected from the medical students rotating through the department of Haematology. Exclusion criteria for the healthy volunteers included: the use of medication which could potentially influence the INR (e.g. vitamin K antagonists), any known underlying liver disease or coagulation factor deficiency and a haematocrit of less than 25% or more than 55%.¹²

Materials and Methods

An experimental study was performed to compare INR values obtained by processing coagulation specimens containing different volumes of whole blood.

Whole blood was collected from each subject via standard venipuncture of the antecubital vein. Respectively 2.5ml, 3 ml, 3.5 ml, 4 ml and 4.5 ml of whole blood was drawn into separate evacuated silicone covered glass tubes containing 3.2% (0.109M) sodium citrate (Becton Dickinson (BD) Vacutainer™ REF 367714). The resulting ratios of whole blood to sodium citrate were: 5:1 (56%), 6:1 (67%), 7:1 (78%), 8:1 (89%) and 9:1 (100%) respectively. The fill volume sequence for each study subject was determined randomly.

The fresh citrated blood samples were centrifuged at 3 000 g for ten minutes. These samples were subsequently maintained at room temperature and the INR determined within 4 hours of venipuncture.

The prothrombin time was measured on the platelet poor plasma using the BCT autoanalyser (Dade Behring) and the Thromborel S (Dade Behring) thromboplastin reagent which consists of human placental thromboplastin (International Sensitivity Index [ISI] = ± 1.00) and contains calcium chloride.

The INR of each collection tube was automatically calculated by utilizing the ISI assigned to the thromboplastin reagent.

The haematocrit (packed cell volume) of each subject was measured using blood collected in a separate EDTA evacuated tube (Becton Dickinson (BD) Vacutainer™ REF 368861). The Advia 120 (Bayer) and the LH 500 (Beckman Coulter) multi channel analysers were used interchangeably to determine the haematocrits of the study subjects.

The study protocol was approved by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, and all subjects gave their written informed consent.

Statistical Analysis

Basic descriptive analysis was undertaken to compute the mean, mode, median and the associated measures of central tendency (standard deviation, standard errors and coefficient of variability) for the variables on both the patients on oral anticoagulant therapy and the healthy volunteers.

The data for the patients on oral anticoagulant therapy and the healthy volunteers were subsequently combined. The combined data were subjected to further analysis using general linear models (analysis of variance, and covariance) using STATA 9 after conversion from Microsoft Excel™ format in order to evaluate the significance of various factors, namely the different tube fill volumes, gender, age and group (patients vs. healthy volunteers) on each of the parameters of interest (PT and INR). Two tailed p-values < 0.05 were considered statistically significant.

Results

The oral anticoagulant group consisted of 19 female (63%) and 11 male (37%) patients with ages ranging from 22 to 79 years. The indications for oral anticoagulation therapy varied and included both therapies for thromboembolic events as well as secondary prophylaxis.

The INR values increased with progressive reduction in the total tube fill volumes. However, the different tube fill volumes did not have a statistically significant influence on the INR (Table 1). The INR values of men were significantly higher compared to those of women ($p = 0.0362$).

Analysis of covariance showed differences between gender ($p = 0.0044$), a significant effect of the use of age ($p = 0.0000$) as a covariate and also weekly warfarin dose ($p = 0.0428$) to be associated with variation in INR values. The prothrombin times were consistently longer and the INR values were consistently higher in male patients when compared to female patients. In contrast, the influence of the other two independent variables on prothrombin time and INR were inconsistent.

Based on the analysis of the study results, tube fill volumes did not influence results significantly compared to the mean and 2 standard deviations calculated from the completely filled blood tubes (100%) in the patients using oral anticoagulant therapy.

INR values obtained for patients taking oral anticoagulant therapy had larger standard deviation, which indicated low level of accuracy or poor control on warfarin therapy.

The standard error of the mean (SEM) is shown in Table 2, together with the prothrombin times and INR values of the patients using anticoagulant therapy. The tendency of INR values to increase as tube fill volumes decrease is illustrated in Figure 1.

The healthy volunteer group consisted of 9 female (60%) and 6 male (40%) patients. As could be expected, the healthy volunteers were significantly younger than the patients on oral anticoagulant therapy (mean 24 vs. 56 years; $p = 0.02$).

The results for the healthy volunteer group showed that different tube fill volumes up to a minimum of 3ml (55%) did not have a significant influence on INR values (Tables 3).

Gender affected the results significantly with male volunteers having higher INR values when compared to female volunteers (Figure 2).

Results for prothrombin times and INR values in healthy volunteers at different total fill volumes are given in Table 4.

Consistent with the results obtained for patients taking warfarin therapy, findings in healthy volunteers confirmed that PTs and INR values tended to become progressively longer as tube fill volumes decreased (Figure 3). However, this prolongation was shown to be more pronounced in patients taking oral anticoagulant therapy.

Discussion

To ensure reliable and accurate laboratory results requires an appreciation of variables which may influence the test results at the time of specimen collection, transport, preparation and analysis.

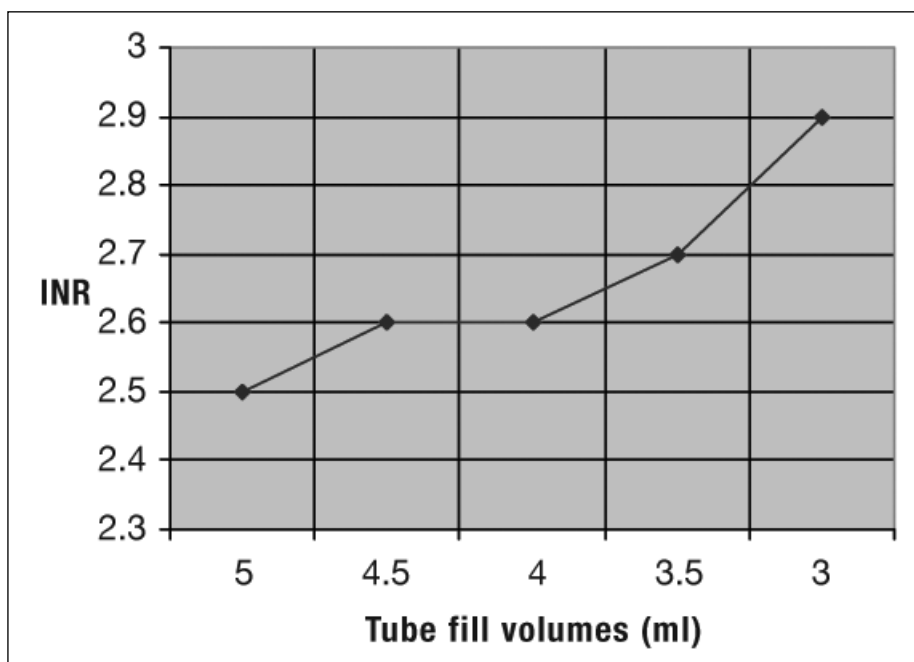


Figure 1: Plot of INR by tube fill volumes for subjects taking warfarin

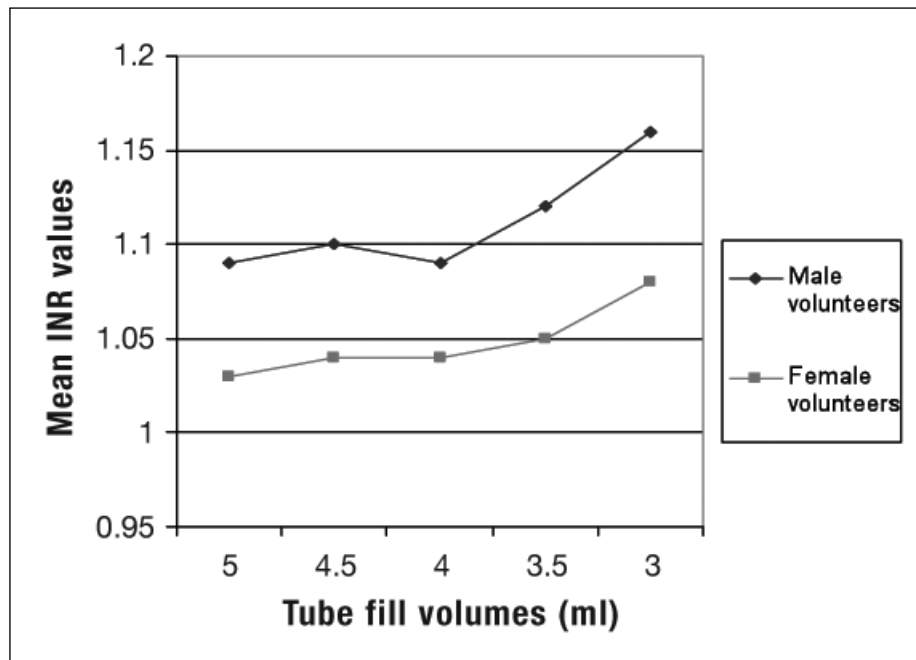


Figure 2: Plot of INR by tube fill volumes for healthy male and female volunteers

In this research project the total tube fill volume was assessed as an independent variable in INR determination in healthy volunteers and in patients who are on oral anticoagulant therapy. The aim was to establish at which total tube fill volume the INR could no longer be determined reliably. This has practical application since specimens submitted for coagulation studies are still regularly rejected due to intrinsic specimen quality requirements.

The responsibility of providing guidelines for rejection of unsatisfactory specimens remains that of the laboratory. Rejecting fewer blood specimens submitted for coagulation testing, especially from INR clinics which still use whole blood testing, may reduce the total cost, time lost, frustration and inconvenience to not only the patients, but also the health care workers and laboratory personnel. However, acceptance and processing of samples should be weighted against the quality of the results obtained especially when a drug with a narrow therapeutic index such as warfarin is used.

Citrate inhibits clot formation by binding to the available calcium in a plasma sample. In addition, the calcium contained in the reagents used to perform prothrombin time tests is also bound by excessive citrate present in the blood collection tube. The INR value may be prolonged by increased citrate concentration since more calcium is bound by the anticoagulant and less is available for clot formation.^{7,13} The anticoagulant concentration can be increased by either using a higher concentration of citrate anticoagulant or by a reduction in the plasma volume which may be due to either underfilling of the collection tube or an elevated packed red cell volume. To prevent erroneous results in coagulation studies, guidelines have been published by various authoritative bodies like the NCCLS which recommends that collection tubes filled to less than 90% of the total tube fill volume or blood collected from patients with haematocrits exceeding 55%, be rejected.⁶

Previously, Adcock and co-workers reported that underfilled tubes were consistently associated with prolonged clotting times when compared to completely filled tubes.¹⁴ They could not demonstrate a statistically significant difference in PT results from a 3.2% citrate tube between fill volumes of 60% and 100%.¹⁴ The influence of underfilled tubes on clotting times was shown to be more pronounced in patients taking oral anticoagulant therapy compared

to healthy volunteers.^{2,14,15} It has been suggested that should results obtained from an underfilled tube fall within the reference range, the true value would likely be normal.^{14,16,17} At the same time, a prolonged value obtained from an underfilled tube warranted a repeat test on a completely filled tube.

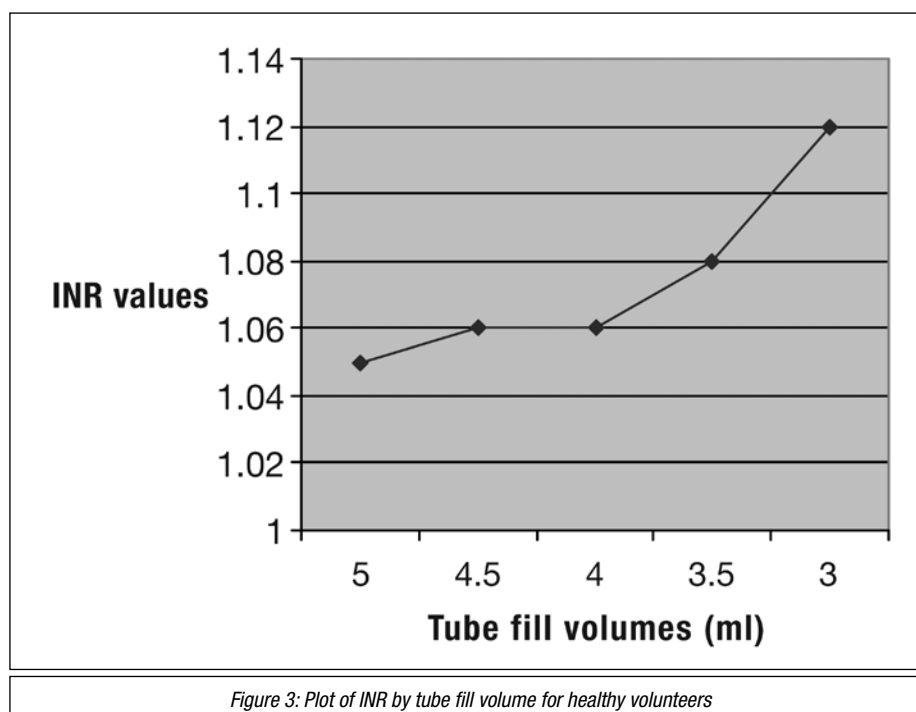
In contrast, Chung and colleagues showed statistically significant INR elevations for sample tube fill volumes of less than 90% in patients on OAT.² They used paediatric (2.5ml) collection tubes to conduct their study. It is evident that the results obtained for specimens collected in paediatric (2.5ml) tubes could not be extrapolated to adult collection tubes.

In the trial conducted by Ingram and Hill, two of three laboratories reported statistically significant lengthening of the prothrombin time, in the plasma of anticoagulated patients, with rising citrate concentrations ($p < 0.01$).¹⁸ This finding was not noted when plasma of healthy individuals was analysed. In subsequent experiments one of the two laboratories which had reported lengthening of the PT, failed to confirm the findings using the same reagent as in the original study.

Reneke and his co-workers showed that accurate PT values could be obtained from normal specimens if the tubes were filled to 65% or more of capacity.¹⁷ Accurate PT results in the therapeutic range, however, were obtained only with filling to 80% or more of capacity (using a "moderately sensitive" thromboplastin reagent – ISI index = 2.06) or 90% or more of capacity (using a "highly sensitive" thromboplastin reagent – ISI index = 1.01).

The findings of this study were consistent with some observations published in the literature in that both patients on oral anticoagulation therapy and healthy volunteers demonstrated progressively prolonged prothrombin times and INR values with decreasing total tube fill volumes.^{2,14} However, this finding did not reach statistical significance in either group for the total tube fill volumes studied. The prolongation of prothrombin times, and subsequently also INRs, was shown to be more pronounced in patients taking oral anticoagulant therapy.

Although the protocol of this research project anticipated and endeavored to prevent inclusion of poorly controlled patients on oral anticoagulant



therapy, prothrombin times and INR values obtained for patients taking oral anticoagulant therapy were still widely spread. This observation indicated poor control of patients on warfarin therapy. These values were responsible for the wide standard deviation. Square root transformation of the data did not alter the findings.

An unexpected and surprising finding was the effect of gender on both the PT and INR values. Male gender was consistently associated with longer prothrombin times and higher INR values in both the healthy volunteers and the patients on oral anticoagulant therapy. This feature has not been described in the literature studied. It is possible that higher estrogen levels in female patients cause the relatively reduced INR values. The male sex was found to be an independent predictor of severe bleeding in patients taking oral anticoagulant therapy.^{19, 20} The higher INR values observed in males in this study may be related to this finding. Although aspects relating to co-morbidity or lifestyle may contribute to this feature, the explanation remains unclear and warrants further investigation.¹⁹

Analysis of covariance showed age (in both groups) and weekly warfarin dose (in patients on oral anticoagulation) to be associated with significant variation in prothrombin times and the INR values. The influence of these two independent variables on prothrombin time and INR were inconsistent and could therefore not be considered to be independent factors in terms of their influence on the PT and INR.

Conclusion

This study reconfirmed that the prothrombin times were prolonged and the INR values were higher when these tests were performed on underfilled collection tubes containing 3.2% citrate anticoagulant. However, for blood specimens collected in 3.2% citrate anticoagulant using our reagent and instrument combination, a total tube fill volume of greater than 56% yielded reliable results. This finding was applicable to both patients on OAT as well as healthy volunteers.

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