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Monitoring the ingestion of anti-tuberculosis drugs by simple non-invasive methods

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Abstract

This investigation retrospectively assessed inexpensive non-invasive qualitative methods to monitor the ingestion of anti-tuberculosis drugs isoniazid, rifampicin and rifapentine. Results showed that commercial test strips detected the isoniazid metabolites isonicotinic acid and isonicotinylglycine as efficiently as the isonicotinic acid method in 150 urine samples. The presence of rifamycins in urine samples ($n = 1085$) was detected by microbiological assay techniques and the sensitivity compared to the *n*-butanol extraction colour test in 91 of these specimens. The proportions detected by the two methods were significantly different and the sensitivity of the *n*-butanol procedure was only 63.8% (95% CL 51.2–76.4%) as compared to that of the superior microbiological method. Final validation ($n = 691$) showed that qualitative assays measure isoniazid and rifamycin ingestion with an efficiency similar to high-performance liquid chromatography. The qualitative procedures may therefore be valuable in clinical trials and in tuberculosis clinics to confirm drug ingestion.
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1. Introduction

There have been limited advances in the treatment of tuberculosis, which severely limits current therapeutic options. This is of great concern in view of the increasing incidence of drug resistant *Mycobacterium tuberculosis*, which is usually the consequence of inadequate therapy. Non-adherence and malabsorption of anti-tuberculosis drugs are fundamental causes of insufficient treatment and the reason for sub-therapeutic serum concentrations and hence poor clinical response (Chan and Iseman, 2002; Kimberling et al., 1998; Mehta et al., 2001). Lengthy dosing regimens with multiple drugs increase the risk of non-adherence, especially if therapy is not supervised. Directly Observed Therapy (DOT), which is used to enhance patient adherence (Chan and Iseman, 2002), may involve measures for confirming drug ingestion by testing for the presence of the medication or its metabolites in the serum or urine of patients (Peloquin, 2002; Meissner et al., 2002). High-performance liquid chromatography (HPLC) assays (Smith et al., 1999; Seifart et al., 1995; Langdon et al., 2004) are available for accurate serum drug concentration determinations, but these are expensive, labour intensive and require a well-equipped laboratory with trained personnel. Although HPLC is essential for pharmacokinetic measurements, under certain conditions it could be too invasive, too complicated and impractical for continuous and rapid monitoring of adherence. An alternative option for monitoring regular drug intake is to make use of inexpensive, non-invasive qualitative urine tests which detect specific drugs and their metabolites (Elizaga and Friedland, 1997; Mitchison and Allen, 1970; Liu et al., 1999; Whitfield and Cope, 2004).

Progress in current anti-tuberculosis drug research ultimately requires clinical trials to measure the action of new candidate compounds in patients with tuberculosis. Studies on the early bactericidal activity (EBA) of anti-tuberculosis drugs have

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shown that patients do not always take their prescribed medication as scheduled during clinical trials (Donald et al., 2003; Sirgel et al., 2000). Drug monitoring during these trials is therefore needed to verify that all participants adhere to therapeutic instruction. Qualitative methods, in contrast to HPLC assays, do not discriminate between parent compounds and their metabolites, but are suitable to verify correct drug ingestion, which might result in more accurate clinical trials.

This investigation retrospectively evaluates urine assays that were used to monitor patient adherence to prescribed therapy schedules in EBA studies (Donald et al., 2003; Sirgel et al., 2000). Commercial test strips and an isonicotinic acid method were compared for the detection of isoniazid (INH) metabolites. The presence of the rifamycins RMS, rifampicin (RIF) and rifapentine (RPT) and their desacetyl metabolites was determined by a microbiological plate diffusion assay and compared to an *n*-butanol colour test. Finally, the isonicotinic acid and microbiological assays were validated against an HPLC method by examining urine samples from patients who received antituberculosis drugs.

2. Materials and methods

2.1. Materials

Isonicotinic acid (Sigma), rifampicin (Novartis, Spartan) and rifapentine (Hoechst Marion Roussel, Inc. mfd by: Gruppo Lepetit S.p.A.) were used in the study. Sodium acetate anhydrous, barbituric acid and acetone were supplied by MERCK. Potassium cyanide was obtained from NT Laboratory Supplies and chloramine-T from SAARCHEM (Pty) Ltd., RSA. *Staphylococcus aureus* strain NCTC 10702, which is susceptible to RIF, but resistant to various other drugs (1000 μg/ml streptomycin, kanamycin, viomycin and capreomycin as well as 500 μg/ml cycloserine) and a RIF-resistant variant of this strain NCTC 10703, were used as indicator organisms in the plate diffusion assay (Mitchison and Allen, 1970).

2.2. Patients and clinical specimens

Patients were derived from two EBA studies and one non-EBA study.

2.2.1. EBA study 1

In this study, 52 patients were recruited for treatment with a specific dose of streptomycin on 2 consecutive days (Donald et al., 2002). Commercial test strips, MYCODYN URITEC^{TM} from DynaGen, Inc., Cambridge, MA, USA (Pekovic et al., 1998) were used in addition to the isonicotinic acid method (Ellard and Greenfield, 1977) to monitor urine samples for the presence of INH metabolites. Both methods are designed to detect the INH metabolites, isonicotinic acid and isonicotinylglycine in urine.

2.2.2. EBA study 2

For this study, 57 patients were enrolled to compare the early bacterial activities of a range of dose sizes of two RMS: RIF and RPT (Sirgel et al., 2005). Patients received either daily doses of 150, 300 or 600 mg RIF for 5 days or one dose of 300, 600, 900 and 1200 mg RPT. Urine (15 ml) samples were collected on 2 consecutive days before receipt of study drugs, followed by a daily collection for 5 days, at 24 h intervals after the intake of a particular dosage. The urine samples were then screened with the isonicotinic acid method for the presence of INH metabolites, and the microbiological method (Mitchison and Allen, 1970) for the RMS. A comparison was made between the microbiological assay and the *n*-butanol colour test (Burkardt and Nel, 1980) for the RMS and its desacetylated metabolite on 91 urine samples collected from the first 13 patients.

2.2.3. Study 3

In this non-EBA study, the isonicotinic acid and microbiological assays were validated by examining 691 urine samples of which the INH, RIF, RPT and metabolite concentrations had been determined by HPLC technology (Langdon et al., 2004). The study was conducted at the DP Marais Hospital in Cape Town. Patients enrolled in the study had been receiving standard anti-tuberculosis therapy (RIF, INH, pyrazinamide, ethambutol and/or streptomycin) for not less than 4 weeks and not more than 6 weeks. Urine collections for HPLC analysis of RIF total concentration were made at 2 h intervals (0–2, 2–4, 4–6 and 6–8) for a total of 8 h on two occasions, separated by at least 3 days. Urine samples for the HPLC determination of total RPT concentration were collected on a further two occasions following a single 15 mg/kg dose of RPT. Each occasion was separated by 4 days. Collections were made at 2 h intervals (0–2, 2–4, 4–6 and 6–8) for a total of 8 h. After HPLC analyses, the urine samples were made available for screening by isonicotinic acid and microbiological assays for the presence of the INH metabolites and rifamycins with their specific metabolites. Each urine sample was tested for the presence of the relevant rifamycin. The three specimens collected during the 2–8 h period from each individual were then pooled and tested for the presence of INH metabolites with the isonicotinic acid method. These findings were qualitatively compared to those obtained with HPLC.

2.3. Assays

2.3.1. Test strips

Each MYCODYN URITEC^{TM} test strip (7.5 cm × 0.5 cm of absorbent paper) is impregnated with barbituric acid, potassium thiocyanate, citric acid and chloramine-T. To detect INH metabolites, the test strip is placed with the barbituric end first into 0.5–1 ml of urine contained in a test tube (13 mm × 100 mm). As the urine diffuses upwards into the test strip, acidified potassium thiocyanate mixes with chloramines-T and as a result cyanogen chloride is produced. Cyanogen chloride then reacts with isonicotinic acid and isonicotinylglycine to form a glutaconaldehyde derivative which reacts with barbituric acid to give a blue–purple colour within 15 min, if the test is positive. A negative result is indicated by no colour change. However, an orange colour may sporadically appear owing to the use of tobacco or therapeutic amounts of niacin and should be considered as a negative reaction.

2.3.2. Isonicotinic acid method

For the isonicotinic acid method (Ellard and Greenfield, 1977), 0.5 ml urine was pipetted into small transparent test tubes together with 0.2 ml 4 M pH 5.0 acetate buffer. At 15 s intervals 0.1 ml 10% aqueous potassium cyanide, 0.1 ml 10% aqueous chloramine-T and 0.5 ml 1% barbituric acid in acetone/water (1:1, v/v) were added. A blue colour emerges within 30 min only if isonicotinic acid and/or isonicotinylglycine are present as opposed to no colour change in the control urines, which lack INH metabolites. The sensitivity of the two methods was compared by testing for the presence of isonicotinic acid in deionised water at concentrations of 50, 5, 0.5, 0.25 and 0.125 μg/ml, respectively.

2.3.3. Microbiological assay

A microbiological assay (Mitchison and Allen, 1970) was employed to demonstrate the presence and concentration of RIF and RPT in urine. Two assay discs with a diameter of 6 mm (2668; antibiotica-testblättchen from Schleicher & Schuell) were dipped into urine and then separately placed onto two Columbia agar base plates (Biolab; Merck ART No: C36), respectively, seeded with a RIF susceptible (NCTC 10702), and a RIF resistant strain (NCTC 10703) of *Stapylococcus aureus*. In the presence of a rifamycin, a zone of inhibition develops on the test plate which contains strain NCTC 10702, while no activity is expected on the RIF resistant control plate with strain NCTC 10703. Zone formation on both test and control plates indicates the presence of a different or additional anti-microbial agent(s). Inhibition zones were measured with a calliper and compared to the drug dose.

2.3.4. n-Butanol colour test

The *n*-butanol colour test for RMS and its desacetyl metabolite (Burkardt and Nel, 1980) involves the mixing of 10 ml urine with 2 ml *n*-butanol in a test tube by gently inverting the tube twice. The mixture is then allowed to stand for at least 30 s for the butanol to separate. The presence of a rifamycin is indicated by the occurrence of an orange-red colour in the upper butanol layer.

2.4. Statistical methods

The association of activity of the rifamycins in urine as measured by zone diameter and drug dose was examined by regression analysis. The zone diameter versus time data of every individual were summarized in the coefficients of a quadratic curve fitted by the method of least squares. Comparisons of dose levels were made in terms of the mean coefficients of the fitted curves. Coefficient b_0 represents the mean size, b_1 the linear component of the trend and b_2 its quadratic component. The McNemar test was used in examining the association between the microbiological and *n*-butanol assays.

3. Results and discussion

In the first EBA study, only one patient (1.9%) was found to be exposed to INH treatment before the start of the study and was therefore not allowed further participation. The remaining 51 eligible patients received only streptomycin at different dosages for 2 consecutive days and since no other anti-tuberculosis drug was allowed to be taken, the patients were monitored for INH ingestion. One day after the first dosage was given, no INH metabolites were detected, but 24 h after the second dosage, 10 (19.6%) patients were identified with INH metabolites in their urine, confirming unauthorised ingestion of INH. From 150 urine specimens, INH metabolites were independently detected in the same 12 samples by the commercial MYCODYN URITEC™ test strips and the isonicotinic acid method. A separate investigation with serially diluted isonicotinic acid in deionised water showed that the isonicotinic acid method is slightly more sensitive than the commercial test strips: the lowest measurable concentrations of the metabolite by these two methods were 0.2 and 0.50 μg/ml, respectively. Commercial test strips, although expensive, require minimal time and materials to perform and can be used for direct monitoring of patients at clinics. In-house test strips (Meissner et al., 2002; Kilburn et al., 1972) can easily be prepared with similar sensitivity and specificity as commercial products, at an affordable price for use in developing countries. The isonicotinic acid method on the other hand, needs to be carried out in a laboratory and involves the preparation and handling of hazardous reagents. Test strips (Kilburn et al., 1972) and laboratory tests (Schraufnagel et al., 1990; Ellard and Greenfield, 1977), which are based on similar chemical reactions have recently been modernised into rapid and safe point-of-care testing procedures for the detection of INH in urine. These developments have resulted in easy-to-use INH urine test strips (BD BBL™ Taxo™ and MYCODYN URITEC™) and the IsoScreen device (Surescreen Diagnostics Limited, Derby, UK) (Whitfield and Cope, 2004), which can be used by non-laboratory personnel in outpatient clinics. A major benefit of point-of-care testing lies in the speed at which a result can be generated, since there are no delays due to the transportation of the specimen to the laboratory and the subsequent transmission of results to the clinic. The immediate availability of results allows direct counselling of the patient if needed. IsoScreen and test strips also have the advantage that the user is protected from dangerous chemicals and potentially infected urine samples.

In the second study, urine samples ($n = 394$) were collected from 57 patients and tested for the presence of INH metabolites and the RMS. In only 1 (1.75%) of these patients, INH and RIF were detected in both the pre-treatment samples. The following five urine samples, from each of the remaining 56 patients, showed no sign of INH ingestion, indicating that unauthorised anti-tuberculosis drugs were not taken while mono-therapy with a rifamycin was administered. The microbiological assay measures antibacterial activity in urine and the diameter of the inhibition zones is therefore associated with drug concentrations which are dose dependent (Figs. 1 and 2).

3.1. Rifampicin

The mean values of b_0 are shown in the last column of Table 1. They are: 13.40 mm (150 mg), 15.89 mm (300 mg) and

Table 1
Anti-microbial activities (zone sizes) in urine of patients who received either a daily dosage of rifampicin for 5 days or a single dose of rifapentine, within a range of dose sizes

Drug	Dose (mg)	No. of patients	Mean zone diameter (mm)					Mean
			Day 1	Day 2	Day 3	Day 4	Day 5	
Rifampicin daily doses	150	7	13.57	13.86	13.57	13.57	12.43	13.40
	300	9	15.66	16.11	16.88	16.44	14.33	15.89
	600	8	23.62	24.37	22.87	20.87	18.37	22.03
Rifapentine single dose	300	7	20.14	17.29	12.71	6.29	3.29	11.94
	600	9	21.67	19.00	15.22	10.11	5.78	14.36
	900	8	24.75	22.63	15.88	9.00	5.43	15.54
	1200	8	24.88	23.38	21.13	13.75	7.75	18.18

No zone formation (0 mm) was observed in the two urine samples that were collected from each participant, prior to treatment, confirming the absence of rifamycin in the urine.

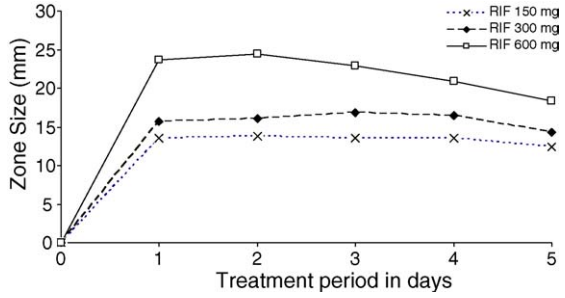

Fig. 1. Presence of rifampicin in urine as indicated by a microbiological assay (zone size), 24 h after each of 5 daily drug doses.

22.03 mm (600 mg). These means differ significantly with a one-way analysis of variance test giving $F(2,21) = 7.98$, $P = 0.003$. A plot of mean b_0 against dose indicates a linear relationship between these two variables, and a regression of b_0 on dose gives a slope coefficient of 0.0194 with standard error 0.00476. The ratio of coefficient to standard error is $t(22) = 4.08$, $P < 0.0005$. One-way analysis of variance tests of difference of the means of the other coefficients reveal no significant differences between dose levels. The apparent curvature of the trend graphs in Fig. 1 is significant. The mean b_2 values of the three dose groups are not significantly different, $F(2,21) = 0.68$, $P = 0.515$. Pooling the b_2 values of the groups gives an overall mean $b_2 = -0.3899$ and testing it for significance of difference from zero gives $t(23) = -3.46$, $P = 0.002$. The decrease in mean zone diameter within the same RIF dose groups over the 5-day treatment period ranged from 13.57 to 12.43 mm (1.09-fold) in the 150 mm group, 15.66–14.33 mm (1.09-fold) in the 300 mg group and 23.63–18.37 mm (1.29-fold) in the 600 mg group (Table 1). This was most evident in the 600 mg group and suggests a reduction in concentration over time, even though the drug was administered daily. The decrease in drug concentration during continuous administration of RIF is almost certainly due to the induction of hepatic enzymes, which leads to auto-induction of its own metabolism (Acocella, 1978).

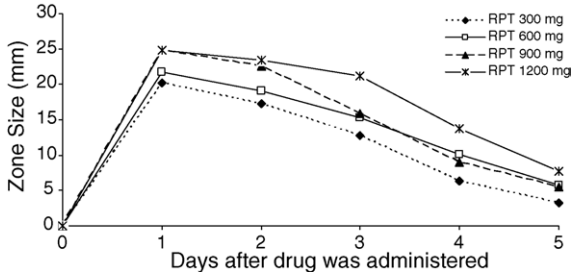

Fig. 2. Presence of rifapentine in urine at five 24 h intervals after a single dose of the drug was administered and measured with a microbiological assay (zone size).

3.2. Rifapentine

The mean values of b_0 are shown in the last column of Table 1. They are: 11.94 mm (300 mg), 14.36 mm (600 mg), 15.54 mm (900 mg) and 18.18 mm (1200 mg) over a 5-day period following a single dose of the drug. A one-way analysis of variance test of homogeneity of these means yields $F(3,28) = 2.13$, $P = 0.118$. However, as in the case of RIF, a plot of mean b_0 against dose indicates a linear relation between these two variables, and a regression of b_0 on dose gives a slope coefficient of 0.00666 with standard error 0.00241. The ratio of coefficient to standard error is $t(30) = 2.77$, $P = 0.010$. One-way analysis of variance tests of difference of the means of the other coefficients reveal no significant differences between dose levels.

The downward trends seen in Fig. 2 are significant. The mean b_1 values of the four groups are not significantly different, $F(3,28) = 0.68$, $P = 0.570$. Pooling the b_1 values of the groups gives an overall mean $b_1 = -4.5062$ and testing it for significance of difference from zero gives $t(31) = -4.51$, $P < 0.001$. The decrease in zone size is obviously due to the fact that the drug was given only once at the beginning of the study and a concentration drop (over time) was therefore expected. Within the RPT doses, the decrease in 300 mg was 6.12-fold; in 600 mg, 3.75-fold; in 900 mg, 4.56-fold and in 1 200 mg, 3.21-fold.

The *n*-butanol test for RMS and their desacetyl metabolites were assessed in the first 13 patients recruited for the second study and compared to the microbiological assay. A rifamycin was detected in 58 of the 91 urine samples with the microbiological procedure, whilst the *n*-butanol method indicated that the drug was present in only 37 of the specimens. However, the two methods agreed that the 26 pre-treatment samples were free of any RMS. From the 65 post-drug samples, the microbi-

Table 2
A comparison between assays for the detection of rifamycins in urine

Assays	*n*-Butanol		Total
	Positive	Negative	
Microbiological			
Positive	30	28	58
Negative	7	26	33
Total	37	54	91

ological and *n*-butanol assays, respectively, tested negative for the presence of a rifamycin in 7 and 28 of the urine specimens (Table 2). All the samples from two patients tested negative for the ingestion of a rifamycin with the *n*-butanol test, although the drugs were given under supervision and their presence was confirmed by the microbiological assay and an HPLC test (Sirgel et al., 2005). The proportions detected by the two methods, 58/91 versus 37/91 are significantly different according to the McNemar test applied to the data in Table 2. The test results in a chi-squared statistic of 12.6 on 1 d.f., $P < 0.001$. The sensitivity of the *n*-butanol test was determined relatively to that of the microbiological assay as 63.8% (95% CL 51.2–76.4%). This finding therefore suggests that the *n*-butanol assay is not suitable for monitoring the ingestion of the RMS and corresponds with an earlier report which indicates that it is unreliable owing to its low sensitivity (64%) and specificity (54%) (Meissner et al., 2002). The microbiological test results (well-defined zones of inhibition) were easy to interpret in contrast to those obtained with the *n*-butanol assay, where a positive result was based on the appearance of a red-orange colour, which fades as the drug concentration drops.

Study 3 compared the sensitivity of the microbiological and isonicotinic acid methods for the detection of the RMS and INH metabolites in urine to that of HPLC. A total of 691 urine specimens was collected from 46 patients at 2 h intervals within the initial 8 h after treatment started, and evaluated for the presence of RIF and RPT. A 90.7% agreement was observed between the HPLC and the microbiological method over the entire 8 h period. Most of the differences occurred during the initial 2 h where only a 73% ($n = 172$) concordance was found. In the remaining 2 h periods (2–4, 4–6 and 6–8 h), the agreement between the two methods was 96.5% ($n = 175$), 97.78% ($n = 174$) and 99.4% ($n = 170$), respectively. The disagreement during the 0–2 h period is probably due to low drug concentrations, since it is expected that the RMS will reach peak concentrations in urine only 2–6 h after ingestion (Kucers, 1997) when a correlation of 96.5–99.4% was achieved. The microbiological method also identified 14 patients suspected of taking drug(s) other than those prescribed for tuberculosis as indicated by zone formation on the RIF-resistant control plates. Further analysis of the case record forms from this study revealed that all of these 14 patients were receiving concomitant doses of cotrimoxazole, which could account for the discrepancy. A 100% correlation was found between the isonicotinic acid and HPLC assays, which indicated that only one patient lacked INH metabolites. All the urine specimens ($n = 4$) that tested negative for INH metabolites were from the same patient. The lack of INH metabolites was explained and confirmed by a clinical report which indicated that it was not administered.

Patients with pulmonary tuberculosis are expected to respond favourably to standard therapy if adequate serum drug concentrations are achieved. However, pharmacokinetic variability in RIF plasma concentrations, with 46% below the suggested normal range, has been observed (Ray et al., 2003; McIlleron et al., 2002). Low RIF serum levels were found to be associated with treatment failure, despite appropriate DOT therapy (Mehta et al., 2001; Van Crewel et al., 2002). Malabsorption of drugs is likely to be the reason for sub-therapeutic serum concentrations and it is therefore suggested that suspect individuals are considered for drug monitoring (Kimberling et al., 1998; Mehta et al., 2001). This investigation indicates the potential of the microbiological assay as a tool for screening large numbers of patients suspected of abnormal RIF plasma levels.

4. Conclusion

The ability of the microbiological, isonicotinic acid and test strip assays to respond correctly in the presence or absence of the RMS or INH in urine justifies their use for monitoring patient adherence. INH and RIF are established members of the standard tuberculosis regimen and their presence in urine can be used as markers to verify drug intake. Furthermore, the capacity of the microbiological assay to distinguish between the RMS levels in urine in relation to dose size emphasizes its quantitative potential to identify patients with sub-therapeutic drug serum levels. Inadequate RIF serum levels, as a result of non-adherence, malabsorption or incorrect dosing, increase the risk of drug resistance and therapeutic failure.

This study has shown that the isonicotinic acid method detects INH metabolites in urine with similar sensitivity as commercial test strips and HPLC. Test strips (Meissner et al., 2002; Pekovic et al., 1998) and the recently described Iso-Screen (Whitfield and Cope, 2004) provide rapid and safe point-of-care testing procedures which overcome some of the limitations associated with the laboratory tests. Point-of-care testing is therefore recommended for use at clinic settings; however, the isonicotinic acid and microbiological assays are valuable alternatives, especially in the context of clinical trials or should the commercial products not be available. This study also demonstrates that the microbiological assay can detect RIF and RPT in urine with considerable sensitivity and accuracy as opposed to the *n*-butanol method, which yielded unreliable results.

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