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Determination of protein-unbound, active rifampicin in serum by ultrafiltration and Ultra Performance Liquid Chromatography with UV detection. A method suitable for standard and high doses of rifampicin

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ABSTRACT

Rifampicin is the most important antibiotic in use for the treatment of tuberculosis (TB). Preclinical and clinical data suggest that higher doses of rifampicin, resulting in disproportionally higher systemic exposures to the drug, are more effective. Serum concentrations of rifampicin are the intermediary link between the dose administered and eventual response and only protein-unbound (free) rifampicin is pharmacologically active. The objective of this work was to develop an ultra performance liquid chromatography assay for protein-unbound rifampicin in serum with ultrafiltration, carried out at a sample temperature of 37 °C, suitable for measurement of concentrations achieved after currently used and higher doses of rifampicin.

Human serum was equilibrated at 37 °C and ultrafiltrated at the same temperature in a Centrifree YM-30 ultrafiltration device, followed by dilution of the ultrafiltrate with methanol and ascorbic acid. Unbound rifampicin was analyzed using ultra performance liquid chromatography with a BEH C18 column, isocratic elution and ultra-violet (UV) detection. The run time was 5 min.

The assay was linear over the concentration range of 0.065–26 mg/L rifampicin in ultrafiltrate. Accuracies for measurement of rifampicin in ultrafiltrate were 97% and 102% at the higher and lower limits of quantitation. Accuracy of the ultrafiltration process cannot be established, as it is not possible to spike blank serum with known amounts of protein-unbound rifampicin. Within- and between-day precision of the method *including* ultrafiltration as well as *after* ultrafiltration were within prespecified limits (CV < 10%). Dilution of the ultrafiltrate with methanol and ascorbic acid kept rifampicin in solution and prevented it from degradation. Rifampicin loss during the ultrafiltration process and variation in analytical results when using two different batches of ultrafiltration devices were both limited. Processed ultrafiltrate samples were stable for 3 days in the autosampler.

The developed method can be applied in pharmacokinetic research, studying exposure-response relationships for rifampicin when administered at higher than currently used doses.

1. Introduction

Rifampicin is one of the pivotal drugs for the treatment of tuberculosis (TB), which is the leading infectious disease in terms of mortality worldwide [1]. A rifampicin dose of 10 mg/kg daily combined with pyrazinamide enabled shortening of the duration of TB treatment to six months decades ago [2]. This 10 mg/kg dose (often 450 or 600 mg, dependent on weight) was chosen in the 1960s and 1970s based on considerations related to pharmacokinetics, cost at the time, and presumed adverse effects [3]. Accumulating data in mice and humans suggest, however, that this 10 mg/kg daily dose is at the lower end of the dose-response curve [4–9]. Our group has performed a dose escalating study in pulmonary TB patients showing that doses up to 35 mg/kg resulted in a more than proportional (ninefold) increase in exposure to rifampicin in plasma, which was safe and well tolerated in small groups [7]. This 35 mg/kg dose of rifampicin combined with isoniazid, pyrazinamide and ethambutol was able to reduce time to sputum culture conversion and may shorten pulmonary TB treatment [8]. A higher dose of rifampicin also reduced mortality in a small study among patients with TB meningitis, the most severe form of TB [9]. Clearly higher doses of rifampicin require more extensive follow-up research, including pharmacokinetic (PK) and pharmacokinetic-

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pharmacodynamic (PK-PD) evaluation.

In pharmacokinetic studies with rifampicin, measurement of the drug in plasma or serum is usually related to the total (protein-unbound plus bound) concentration of rifampicin. An equilibrium between total and protein-unbound concentrations is commonly assumed, yet protein-unbound (free) rather than total drug concentrations are preferably used in concentration-response evaluations [10], as only protein-unbound drugs are pharmacologically active and diffuse (or are being actively transported) into tissues and to the sites of action [11,12]. In a recent pharmacokinetic study with standard dose rifampicin we assessed a two-fold interindividual variation in the unbound rifampicin fraction [13]. This interindividual variability in the free fraction indeed shows that measurement of solely total concentrations could be misrepresentative of the relevant exposure. It is currently unknown what the free fraction of rifampicin is after administration of higher doses that result in much higher rifampicin exposures, possibly associated with saturation of plasma or serum proteins that bind rifampicin. Clearly the redevelopment of rifampicin as a TB drug warrants the development of an analytical method for protein-unbound rifampicin concentrations achieved after administration of high doses of the drug.

Previously we measured free rifampicin concentrations after standard doses using ultrafiltration at room temperature. In ultrafiltration, centrifugal forces are employed as the driving force for the passage of serum water across a filter membrane [14]. The aim of the current project was to develop and validate a new ultrafiltration method for the measurement of the free, active concentration of rifampicin at 37 °C, enabling for PK and PK-PD studies with higher doses of rifampicin.

2. Materials and methods

2.1. Chemicals and materials

Rifampicin (cas.nr. 13292-46-1, purity 98%, see Fig. 1 for chemical structure), acetic acid (100%), ammonium acetate (> 98%), acetonitrile and methanol of LC–MS quality were purchased from Merck (Darmstadt, Germany). Ascorbic acid was purchased from Bufa (IJsselstein, The Netherlands). HPLC quality water was obtained with a Veolia Purelab flex 4 system from Veolia (Ede, The Netherlands). Centrifree YM-30 ultrafiltration filter devices were from Millipore B.V. (Amsterdam, The Netherlands). Drug free human serum was obtained from Sanquin blood bank (Nijmegen, The Netherlands) and was stored at −40 °C.

2.2. Preparation of stocks

Two rifampicin stock solutions containing 1000 mg/L of rifampicin with 200 mg/L ascorbic acid were prepared in methanol: HPLC quality water (4:1%v/v). Ascorbic acid is added to rifampicin to protect it from degradation [15]. Stocks were stored at −40 °C.

2.3. Preparation of calibration stocks for the calibration curve

For the preparation of the calibration curve, one of the rifampicin stock solutions was diluted with methanol to achieve six calibration stocks of 1.3–2.6–7.8–26–78–260 mg/L (calibration stocks 1–6) and were stored at −40 °C until analysis. All stocks were shown to be stable for at least 12 months.

2.4. Calibration curve for unbound rifampicin in serum ultrafiltrate

Calibration curve standards could not exist of serum samples spiked with known amounts of rifampicin that are subsequently being ultrafiltrated, as the analytical method aims to measure unbound concentrations of rifampicin and these concentrations cannot be set (or 'spiked') in serum. Therefore the calibration curve consisted of blank serum ultrafiltrate spiked with rifampicin. Blank ultrafiltrate was obtained by ultrafiltration of drug free human serum.

The calibration curve was made freshly on the day of analysis. First, solutions of rifampicin in blank ultrafiltrate were prepared. For standard 0, 200 µL blank ultrafiltrate was used. For standards 1–6, 10 µL of one of the calibration stocks 1–6 was added to 190 µL blank ultrafiltrate. For standard 7, 20 µL calibration stock 6 was mixed with 180 µL blank ultrafiltrate. The resulting calibration curve concentrations of rifampicin were 0.065–0.13–0.39–1.3–3.9–13–26 mg/L. Secondly, 200 µL volumes of each of these solutions of rifampicin in ultrafiltrate were transferred into maximum recovery® vials (Waters) with 400 µL of methanol: ascorbic acid 20 mg/mL in water (40:1%v/v). The vials were closed with a polypropylene screw cap with silicon/PTFE septum to prevent evaporation. The use of methanol in the samples was pivotal to keep rifampicin dissolved and prevent it from sticking to inserts and vials, whereas ascorbic was essential to prevent disintegration of the drug.

2.5. Internal quality control samples: stocks and sample preparation

Although serum could not be spiked with predetermined protein-unbound concentrations of rifampicin, it was deemed desirable to have internal quality control over the sample preparation procedure. To this end, internal quality control (QC) samples were prepared from the second stock solution (see above) resulting in total (protein-bound plus unbound) concentrations of 1.5, 10 and 61 mg/L in serum, designated as QC Low, Medium and High respectively. The QC samples were stored at −80 °C and found to be stable for at least 26 months.

Sample processing for these QC samples was identical to sample

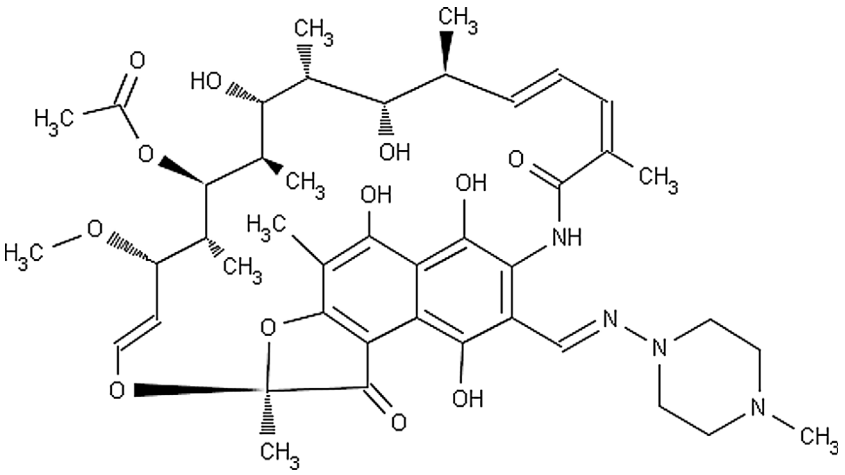

Fig. 1. Chemical structure of rifampicin.