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Note

Measurement of rifampicin and 25-desacetylrifampicin in biological fluids using high-performance liquid chromatography with direct sample injection

S. OLDFIELD, J.D. BERG*, H.J. STILES and B.M. BUCKLEY

Department of Clinical Biochemistry, Sandwell District General Hospital, West Bromwich, West Midlands B71 4HJ (U.K.)

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Rifampicin is an established first-line antituberculous agent derived from rifamycin SV and its use in other serious infections is increasing [1, 2]. It is metabolised in the liver mainly by deacetylation and is excreted with its metabolites in bile. Hence, care must be taken when rifampicin is used in patients with liver disease [3, 4] and in such circumstances monitoring the serum concentrations of rifampicin might be of value in optimising the dose.

Two different techniques have been in general use for the measurement of rifampicin concentrations in physiological fluids, the most widely employed being microbiological plate diffusion assay [5, 6]. Spectrophotometric methods have also been employed for the determination of rifampicin concentrations in urine [7, 8] and serum [9]. However, neither of these approaches distinguish between rifampicin and its principal metabolite 25-desacetylrifampicin since the latter possesses similar antimicrobial activity and spectral characteristics to the parent drug. Furthermore, the spectrophotometric method involves an extraction step and, though it measures rifampicin quantitatively, recovery of 25-desacetylrifampicin is only about 50%. However, in the absence of better techniques, pharmacokinetic studies of the drug have relied on these methods though they are unsatisfactory in failing to distinguish rifampicin and its metabolites [10, 11]. Recently, high-performance liquid chromatography (HPLC) has been used in the estimation of rifampicin in biological fluids [12—16]. This technique has the advantage of resolving rifampicin, 25-desacetylrifampicin and other putative metabolites and is more sensitive than previous analytical methods. However, to date HPLC

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methods have required extensive sample pretreatment and long analysis times. They have all relied on UV detection.

This work describes a technique for the measurement of rifampicin and 25-desacetylrifampicin concentrations in serum and urine. Pre-analytical steps are eliminated by direct injection of untreated sample on to a pre-column, together with a simple column-switching procedure. The use of electrochemical detection in tandem with UV detection enhances sensitivity and specificity. The method is rapid and precise.

EXPERIMENTAL

Samples and standards

Serum standards were prepared by supplementing non-icteric pooled human serum with 12.5 mg/l rifampicin (Sigma, Poole, U.K.) and 0.5 mg/l 25-desacetylrifampicin (a gift from Ciba-Geigy, Basle, Switzerland). Urine standards were aqueous solutions of 40 mg/l rifampicin and 20 mg/l 25-desacetylrifampicin. Standards were stored in 0.5-ml aliquots at −20°C. Patient samples were analysed on the day of collection or stored at −20°C until analysed.

HPLC apparatus

An ACS Model 351 isocratic pump (Applied Chromatography Systems, Macclesfield, U.K.) was employed. Samples were injected using a Model 7125 Rheodyne syringe-loading injector with the sample loop replaced by a 25 × 4.6 mm pre-column packed with Lichroprep RP-18, 25–40 μm particle size (HPLC Technology, Macclesfield, U.K.). The analytical column was a 3-μm Hypersil C_{18}, 150 × 4.6 mm stainless-steel column (Technicol, Stockport, U.K.). A Pye-Unicam 4025 UV detector (Pye-Unicam, Cambridge, U.K.) was used at a wavelength of 334 nm. An LCA 15 electrochemical detector with a glassy carbon working electrode (EDT Research, London, U.K.) was used at an oxidising potential of 0.45 V and sensitivity of 30 nA. The mobile phase was 70% aqueous methanol containing 50 mmol/l potassium nitrate at a flow-rate of 0.7 ml/min at room temperature.

Analytical technique

The pre-column was first washed with 250 μl water to remove mobile phase from the previous run. The sample, 20 μl of serum or 20 μl of a 1:6 dilution of urine in water, was injected without pretreatment on to the pre-column and washed with 500 μl of water, followed by 250 μl of 40% aqueous methanol, to remove protein and other unwanted material. The injection valve was then switched to bring the pre-column into line with the analytical column. To minimise band-spreading sample was loaded to and eluted from the same end of the pre-column.

RESULTS

Polarography

Rifampicin and 25-desacetylrifampicin can be detected electrochemically by oxidation of the hydroquinone moiety. To determine a suitable voltage for

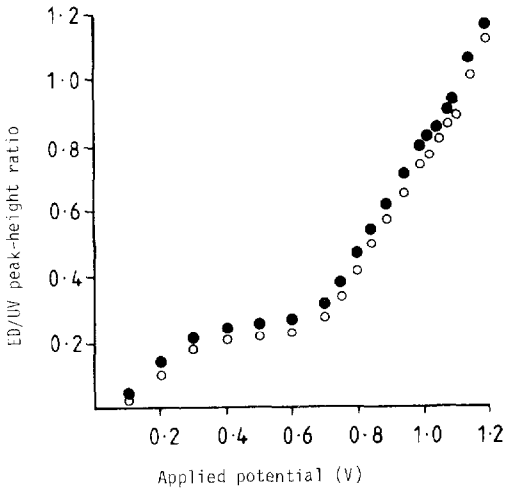

Fig. 1. Polarography of rifampicin (•) and 25-desacetylrifampicin (○). A number of chromatograms of an aqueous solution containing 10 mg/l rifampicin and 5 mg/l 25-desacetylrifampicin were run with different potentials applied across the electrochemical cell. Ratios of peak current to peak absorbance at 334 nm are plotted.

measurement, a series of chromatograms of an aqueous solution containing 10 mg/l rifampicin and 5 mg/l 25-desacetylrifampicin were run at various applied potentials. The ratio of peak current to peak absorbance was plotted (Fig. 1) and a working potential of 0.45 V was selected.

Injection technique

In using a pre-column instead of an injection loop the analytes of interest in a sample can be retained while protein and early-eluting material are washed off to waste. To maintain resolution the analytes should be concentrated in a narrow band on the pre-column packing and not moved by washing solutions, but rapidly eluted by the mobile phase. The binding of rifampicin to and elution from several potential pre-column packing materials were examined qualitatively. Silica C_{18} was found to be best; rifampicin was bound too tightly to the shorter-chain alkyl and phenyl sorbents and formed a diffuse band on cyanopropyl silica. In the present work 20 μl of serum were routinely used but larger volumes can be loaded if pre-concentration of the analytes is required. The pre-column is repacked after approximately 200 injections of serum or urine. Urine samples, which in general contain higher drug concentrations, were pre-diluted with water. A water wash of 500 μl suffices to remove protein from the pre-column. Up to 40% methanol can be used to elute fast-running materials from the pre-column without broadening the rifampicin or 25-desacetylrifampicin peaks.

Linearity

Quantitation was achieved using peak height. The standard curves were linear, using both UV and electrochemical detection, throughout the therapeutic ranges up to 20 mg/l for rifampicin and up to 4 mg/l for 25-desacetylrifampicin. Least-squares linear regression of standard curves yielded

representative equations for the rifampicin line of $y = -0.06x + 0.52$ ($r = 1.000$) and for the 25-desacetylrifampicin line of $y = 0.14x + 0.99$ ($r = 0.996$). Optimisation of the system for different concentration ranges is easily achieved.

Recovery

Rifampicin in serum is predominantly protein-bound. Therefore, for good recovery the pre-column packing must have a much higher affinity for the drug than does protein. A comparison of aqueous and serum standards showed that the method gave 95% recovery of both rifampicin and 25-desacetylrifampicin.

Imprecision

Table I shows the intra- and inter-assay imprecision of rifampicin and 25-desacetylrifampicin at three levels. The between-batch coefficient of variation for rifampicin is less than 5% at the highest concentration tested and less than 10% in each case. The method is optimised to assay therapeutic concentrations, but if the sensitivity of the electrochemical detector is increased to 10 nA, the coefficient of variation at low concentrations falls to less than 5%.

TABLE I

PRECISION OF ASSAY

Rifampicin		25-Desacetylrifampicin	
Concentration (mean ± S.D.) (mg/l)	Coefficient of variation (%)	Concentration (mean ± S.D.) (mg/l)	Coefficient of variation (%)
Within-batch precision (n = 10)			
14.4 ± 0.42	2.9	2.2 ± 0.11	5.0
6.9 ± 0.28	4.0	1.1 ± 0.12	11.6
2.6 ± 0.20	7.8	0.5 ± 0.08	14.6
Between-batch precision (n = 9)			
13.9 ± 0.61	4.4	2.4 ± 0.20	8.5
6.9 ± 0.66	9.5	1.1 ± 0.25	22.5
2.4 ± 0.20	8.3	0.6 ± 0.09	15.0

Patient samples

In the majority of patient samples no interfering peaks were seen on either detector in pre-dose specimens (see Figs. 2B, 3B). However, a few individuals, especially patients with liver disease, showed peaks coincident with either rifampicin or its metabolite on either electrochemical detection or UV detection. No serum or urine specimens were encountered in which interfering peaks were found by both detectors. Use of both electrochemical and UV detection increases the specificity of the method as only peaks that are present on both traces are considered. Fig. 2 shows chromatograms of a standard and of serum samples from a patient on the first day of treatment with 600 mg rifampicin for tuberculosis. A serum sample from this patient taken 8 h after ingestion of the first dose showed clearly-resolved peaks of rifampicin and 25-desacetylrif-

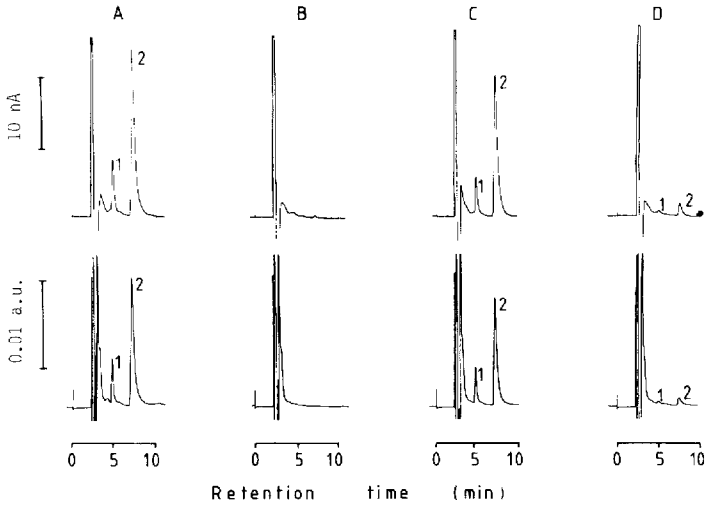

Fig. 2. Chromatograms of rifampicin and 25-desacetylrifampicin in serum, using electrochemical (top row) and UV detection (bottom row). (A) Serum standard containing 2.5 mg/l 25-desacetylrifampicin (peak 1) and 12.5 mg/l rifampicin (peak 2). (B– D) Serum samples from a patient with tuberculosis on the first day of treatment with 600 mg rifampicin daily; (B) pre-dose; (C) 8 h after ingestion of first dose, containing 10.7 mg/l rifampicin and 1.8 mg/l 25-desacetylrifampicin; (D) 24 h after ingestion of first dose, containing 1.0 mg/l rifampicin and 0.1 mg/l 25-desacetylrifampicin.

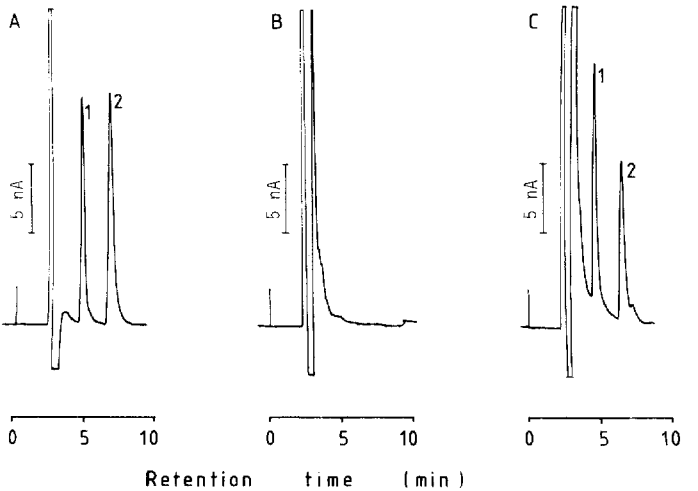

Fig. 3. Chromatograms of rifampicin in urine using electrochemical detection. (A) Aqueous standard containing 20 mg/l 25-desacetylrifampicin (peak 1) and 40 mg/l rifampicin (peak 2). (B and C) Urine from a patient with tuberculosis on the first day of treatment with 600 mg rifampicin daily; (B) pre-dose; (C) 18 h after ingestion of the first dose, containing 26.8 mg/l rifampicin and 20.8 mg/l 25-desacetylrifampicin.

ampicin at concentrations of 10.7 and 1.8 mg/l, respectively (Fig. 2C). Drug and metabolite were still detectable 24 h after the dose. Fig. 3 shows chromatograms of standards and of urine from a patient before and 18 h after ingestion of 600 mg rifampicin. Some fast-running material was present, despite the

pre-column washing procedure, but both rifampicin and 25-desacetylrifampicin were clearly resolved (Fig. 3C).

DISCUSSION

A simple HPLC method suitable for the routine quantitation of rifampicin and its major metabolite, 25-desacetylrifampicin, using direct injection of serum or urine and simple column switching is described. Direct injection of sample can avoid lengthy sample preparation procedures and the imprecision they may introduce, but published methods on direct injection have required complex equipment using more than one injection valve and pump [17]. The technique described here involves simple manual steps using a single injection valve for loading and washing the sample and for column switching. The procedure can be easily automated with a suitable autosampler incorporating a diluter.

Previous HPLC methods for rifampicin determination have used extraction techniques [12- 16], some of which are quite complex and lengthy. Internal standards are required to compensate for potential loss of analyte at each stage. Direct injection is rapid and, since there are no preparative steps during which analyte can be lost, an internal standard is not needed. This is confirmed by the imprecision data: the method has a coefficient of variation of less than 5% at peak therapeutic concentrations and less than 10% at the two lower concentrations of rifampicin tested.

The use of two detectors increases the chemical specificity of the assay as underlying peaks may be seen on one or other detector in a few difficult sera. For example, rifampicin quantitation is difficult in a few patients with liver disease, due to the presence of electroactive material with a similar retention time. Electrochemical detection is more sensitive than UV detection; a sensitivity of 30 nA is suitable for measuring therapeutic concentrations of the drug, but at a sensitivity of 10 nA, 0.05 mg/l rifampicin and 0.02 mg/l 25-desacetylrifampicin can be detected. Even at high sensitivity the electrochemical detector stabilises rapidly and is easy to use. In spite of the use of 70% methanol as mobile phase, the glassy carbon working electrode has been used unchanged for several thousand analyses.

The method is ideally suited to the assay of serum rifampicin and 25-desacetylrifampicin for therapeutic drug monitoring purposes and is also suitable for pharmacokinetic studies. The ability to quantitate rifampicin and its major metabolite independently is essential for pharmacokinetic studies and the high sensitivity attainable enables rifampicin to be detectable for at least 24 h following a therapeutic dose and allows pharmacokinetics of lower dose regimes to be studied.

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