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Simultaneous high-performance liquid chromatographic determination of quinupristin, dalfopristin and their main metabolites in human plasma

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Abstract

Quinupristin–dalfopristin (30:70, w/w) is a new streptogramin, which has been developed for intravenous use. A specific and sensitive HPLC method was developed to measure simultaneously quinupristin (RP 57669) and dalfopristin (RP 54476) and their main metabolites in human plasma. The metabolites measured by this method were RP 69012 (glutathione-conjugated) and RPR 100391 (cysteine-conjugated) from quinupristin and RP 12536 (natural pristinamycin IIA), from dalfopristin. Solid-phase extraction with disposable cartridges was combined with reversed-phase HPLC and fluorimetric detection for RP 57669, RP 69012 and RPR 100391 and UV detection for RP 54476 and RP 12536. The method provided good recovery and low limits of quantitation (0.025 mg l^{-1} for RP 57669, RP 54476 and RP 12536, and of 0.010 mg l^{-1} for RP 69012 and RPR 100391). The validated range of concentrations of the method was: 0.025–5000 mg l^{-1} for RP 57669, RP 54476 and RP 12536 and 0.010–0.750 mg l^{-1} for RP 69012 and RPR 100391. © 1998 Elsevier Science B.V.

Keywords: Quinupristin; Dalfopristin

1. Introduction

The new injectable streptogramin quinupristin–dalfopristin, is a combination of two semisynthetic derivatives, quinupristin (5δ *R*-[(3*S*)-quinuclidinyl] thiomethyl-pristinamycin IA, RP 57669) and dalfopristin (26 (*R*)-(2-diethylaminoethyl) sulphonyl pristinamycin IIB, RP 54476) (Fig. 1) in a fixed 30:70 (w/w) ratio. In the case of individual administration, RP 57669 and RP 54476 have only limited antibacterial activity but synergistically demonstrate marked bactericidal activity against many pathogenic Gram-positive cocci [1,2].

RP 57669 and its metabolites RP 69012 and RPR 100391 are the main quinupristin-derived components which have been identified in human plasma. RP 54476 and its metabolite RP 12536 (natural pristinamycin) are the main compounds encountered in human plasma.

Metabolic pathways are illustrated in Fig. 1. Faecal excretion constitutes the main elimination route for both compounds and their metabolites (75–77% of dose) [3].

In vitro, RP 69012 and RPR 100391 have the

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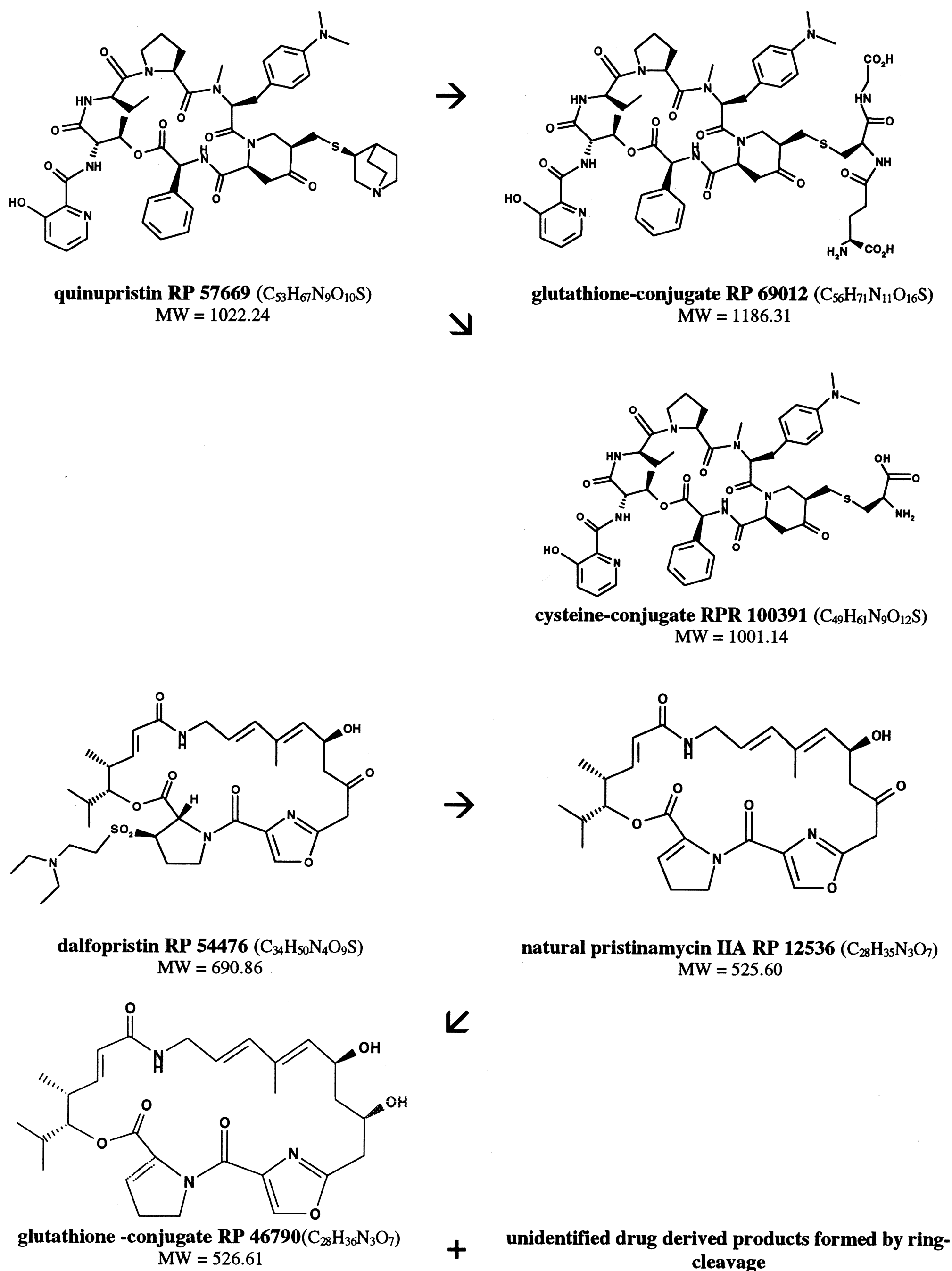

Fig. 1. Metabolic pathways of the streptogramins.

same antimicrobial activity as compared to RP 57669, and RP 12536 has the same antimicrobial activity as RP 54476. The metabolites express a synergistic activity similar to the parent drug, when associated to either RP 57669 or RP 54476.

Ex-vivo, in biological medium, RP 57669 and RP

54476 are spontaneously converted into metabolites via non-enzymatic chemical reactions. The cysteine-conjugated (RPR 100391) and the glutathione-conjugated (RP 69012) derivative of RP 57669 are formed ex vivo in human blood and RP 12536, which is rapidly formed ex vivo following the hydrolysis of RP 54476 in human blood or plasma, is itself unstable in biological medium. pH adjustment to acidic conditions is required for suitable blood sample collection in order to insure the stability of the streptogramins and avoid ex vivo degradation.

A reliable and sensitive method has been developed for the simultaneous measurement of quinupristin, dalfopristin and these three active metabolites in human plasma.

2. Experimental

2.1. Standards and reagents

RP 57669 (quinupristin), RP 54476 (dalfopristin), RP 69012, RPR 100391, RP 12536 (pristinamycin IIA) and the internal standard (RP 51733: dimethylamino-3-propyl) thiomethylene-5 virginiamycin S) were provided by Rhône-Poulenc Rorer (CRVA) France. The structures of the streptogramins are shown in Fig. 1. The compounds were initially dissolved in methanol (for analysis – Merck). Further dilutions were prepared in methanol–water (70:30, v/v) containing 3.5 m*M* of pentane sulfonic acid (PSA) (Waters, Milford, MA, USA). Acetonitrile (HPLC grade) was obtained from Distrilab (Leusden, Netherlands). Perchloric acid and hydrochloric acid (RP Normapur) were obtained from Prolabo (Paris, France) and pH 3 buffer solution from J.T. Baker (Deventer, Netherlands).

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of two type PU 980 JASCO high-pressure pumps, a type 875 JASCO UV and a type 821-FP JASCO fluorimetric detector, all obtained from Prolabo. For calculation of the signal responses a type 4930 ChemServer Data Acquisition System (CAS) from Hewlett-Packard (Palo Alto, CA, USA) was used.

Chromatographic separation was achieved on a Kromasil C_{18} column (125×4.6 mm I.D., 5 μm particles) obtained from Higgins Analytical (Mountain View, CA, USA). The analytical column was protected by a short guard column of μBondapak C_{18} (10 μm particles) from Waters which was routinely changed after 30 injections. The mobile phase consisted of 0.8 ml of 70% perchloric acid (PCA)/l of water (solvent A) and acetonitrile (solvent B). A step gradient was used for sample elution: 30% solvent B for 11 min, 32% solvent B from 11.1 to 15 min, 40% solvent B from 15.6 to 16 min, 38% B from 16.1 to 34 min and 80% B from 34.1 to 36 min.

The percentage of B was then returned to its initial value (30%) for column re-equilibration.

The ASPEC system proceeds to a new injection 9 min after returning to its initial value (30%). The mobile phase was delivered at a flow-rate of 0.5 ml min^{-1} initially for 11 min and was then increased to 1 ml min^{-1} up to a run-time of 36 min. During re-equilibration a flow-rate of 0.5 ml min^{-1} was used.

For fluorimetric detection an excitation wavelength of 360 nm and an emission wavelength of 410 nm was chosen for RP 57669, RP 69012 and RPR 100391. For UV detection, the wavelength was 235 nm for RP 54476 and RP 12536.

2.3. Assay procedures

Solid-phase extraction (SPE) of the five compounds with CN cartridges obtained from Lida-Interchim (Kenosha, WI, USA) was combined with reversed-phase high-performance liquid chromatography (HPLC) in a completely automated analyser. The fully automated Gilson ASPEC XL system was used to develop a procedure for measuring the five compounds in human plasma.

A combination of sodium citrate and hydrochloric acid was used for sample collection in order to avoid clotting and degradation of the parent compounds and their metabolites, since at room temperature RP 57669 and RP 54476 were found to be unstable when only freshly citrated human plasma was used. After 15 min under these conditions, 12% of RP 57669 was transformed into RP 69012 and RPR 100391, and 18% of RP 54476 was transformed into RP 12536.

These preliminary results indicated that blood samples taken during clinical studies have to be acidified immediately when the sample is drawn. A delay of 15 min can lower the concentrations of the parent compounds by 10 to 20% and increase the levels of the metabolites accordingly.

The parent compounds were found quite stable if the following procedure was used: immediately after collection, 10 ml of blood was measured into a tube containing 1 ml of sodium citrate (3.8%) and exactly 2.5 ml of 0.25 *M* hydrochloric acid. The mixture was gently shaken by hand, and tubes were centrifuged at 2000 *g* at +4°C for 15 min. The plasma solution was collected and divided into two aliquots of at least 2 ml and frozen at −20°C. For calculation of the levels of either drug or metabolites, it has to be considered that 1.35 ml of acidified citrated plasma contained 1 ml of plasma.

Plasma samples (1.35 ml of acidified citrated plasma) were prepared for extraction by the successive addition of 1 ml buffer solution (0.085 *M* citric acid monohydrate pH 3–0.081 *M* NaOH–0.060 *M* HCl) and 50 μl of internal standard RP 51733 (100 mg l^{-1}) in methanol. Each sample was shortly vortexed for a few seconds. After centrifugation (5 min 4000 *g* at 4°C) the plasma solution was transferred into a 6-ml glass tube and placed into the ASPEC sample rack thermostatted at +12°C.

The following operations on the sample were performed automatically by the ASPEC system: (1) extraction column conditioning: 1 ml methanol, 1 ml of demineralized water, 1 ml of buffer solution (pH 3). (2) SPE: diluted plasma (approx. 2.5 ml) was dispensed into the extraction cartridge and subsequently dried under positive pressure (3 ml air pushed at a dispensation flow-rate of 0.5 ml min^{-1}). (3) Elution: samples were eluted with 0.5 ml of methanol–water (70:30) containing 3.5 m*M* of PSA.

2.4. Calculations

The Hewlett-Packard Chemserver 4930 software was used to calculate the concentrations of RP 57669, RP 69012, RPR 100391, RP 54476 and RP 12536.

Calibration curves were obtained by weighted linear regression ($1/y^2$) of the peak height ratios of RP 57669, RP 69012, RPR 100391, RP 54476 and RP 12536 versus I.S. under either fluorimetric or UV detection.

Concentrations were calculated by using the slope and the intercept of the calibration lines. Concentrations are reported as mg l^{-1} expressed as free base.

3. Results

3.1. Specificity

The specificity of the method was verified with respect to possible interferences by comparing the chromatograms obtained from drug-free human plasma and human plasma spiked with the five compounds to be analysed prepared from more than five different blood batches. Typical chromatograms of unspiked acidified human plasma and of spiked acidified human plasma are illustrated in Fig. 2 (unspiked plasma, fluorimetric detection), Fig. 3 (unspiked plasma, UV detection), Fig. 4 (plasma spiked with 0.5 mg l^{-1} of RP 57669; 0.25 mg l^{-1} of RP 69012, 0.25 mg l^{-1} of RPR 100391 and 0.5 mg l^{-1} of I.S, fluorimetric detection) and Fig. 5 (plasma spiked with 0.5 mg l^{-1} of RP 54476, 0.5 mg l^{-1} of RP 12536 and 5 mg l^{-1} of I.S, UV detection).

No interferences between the compounds to be analysed and matrix constituents were observed.

The corresponding retention times are 12.7 min (RP 54476), 15.1 min (RP 69012), 16.1 min (RPR

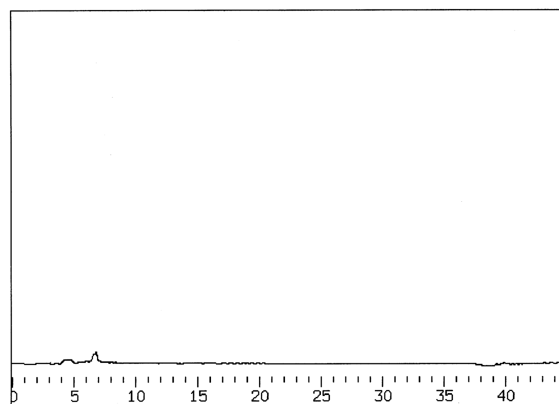

Fig. 2. Typical chromatogram of extracted plasma (unspiked plasma) obtained by fluorimetric detection.

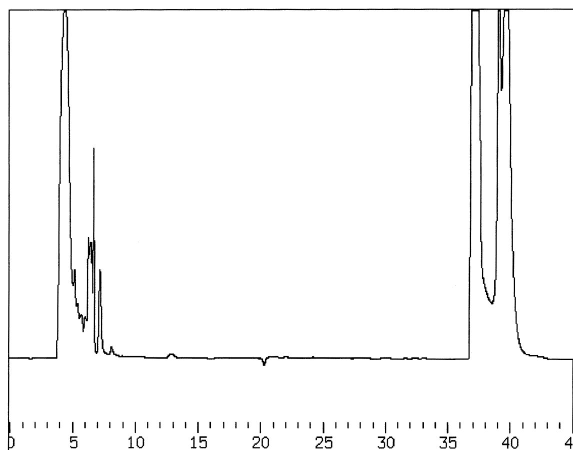

Fig. 3. Typical chromatogram of extracted plasma (unspiked plasma) obtained by UV detection.

100391), 22.1 min (RP 57669), 24.1 min (RP 12536) and 31.0 min (RP 51733).

3.2. Recovery

Recovery of each compound was determined by comparing the average peak heights ($n=6$) obtained from human plasma controls at 0.5 and 2.0 mg l^{-1} for RP 57669, RP 54476 and RP 12536 and at 0.175 and 0.7 mg l^{-1} for RP 69012 and RPR 100391 with

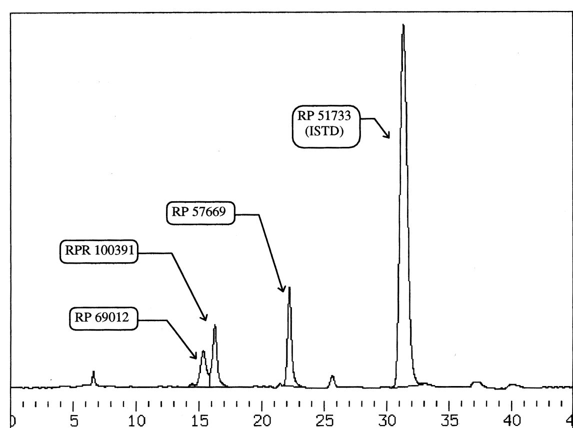

Fig. 4. Typical chromatogram obtained by fluorimetric detection from extracted blood spiked with 0.5 mg l^{-1} RP 57669, 0.25 mg l^{-1} RP 69012, 0.25 mg l^{-1} RPR 100391 and 5 mg l^{-1} RP 51733 (internal standard).

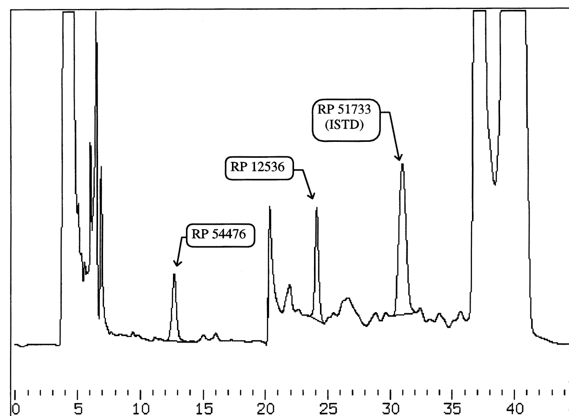

Fig. 5. Typical chromatogram obtained by UV detection from extracted blood spiked with 0.4 mg l^{-1} RP 54476, 0.4 mg l^{-1} RP 12536 and 5 mg l^{-1} RP 51733 (internal standard).

neat standards of equivalent concentrations prepared in 0.5 ml of methanol–water (70:30) containing 3.5 m*M* PSA. The recovery of RP 51733 (internal standard) was determined at 5 mg l^{-1} both for fluorimetric and UV detection. Recoveries were 92.5–94.0% (RP 57669), 100–104% (RP 69012), 111–110% (RPR 100391), 82.9–88.2% (RP 54476), 98.2–101% (RP 12536) and 101% (RP 51733).

3.3. Linearity

Plasma concentrations were linear between 0.025 mg l^{-1} and 5.00 mg l^{-1} for RP 57669, RP 54476 and RP 12536 and between 0.0080 mg l^{-1} and 2.00 mg l^{-1} for RP 69012 and RPR 100391. The measured values for the correlation coefficient r, slope and y-intercept obtained from six replicates at each calibration level are shown in Table 1.

Table 1
Values for the correlation coefficient r, slope and y-intercept for quinupristin, dalfopristin and three of their metabolites obtained from six replicates at each calibration level

Compound	Correlation coefficient, r	Slope	y-Intercept
RP 57669	0.99807	0.6395	−0.0006
RP 69012	0.99554	0.6512	0.0009
RPR 100391	0.99650	1.0269	0.0010
RP 54476	0.99087	1.2849	0.0303
RP 12536	0.99782	2.0063	0.0221

Table 2
Intra-day and inter-day precision and accuracy of RP 57669, RP 54476, RP 12536, RP 69012 and RPR 100391 determination in human plasma

Compound	Spiked concentrations (mg l^{-1})	Precision (%)		Accuracy (%)	
		Intra-day ($n=6$)	Inter-day ($n=34$)	Intra-day ($n=6$)	Inter-day ($n=34$)
RP 57669	0.025	3.7		−7.4	
	0.5	2.5	9.0	−4.3	2.0
	2	1.4	9.7	0.09	2.5
RP 54476	0.025	7.4		12.3	
	0.5	3.3	15.5	−14.4	5.1
	2	4.2	19.4	−8.3	−10.1
RP 12536	0.025	1.6		5.7	
	0.5	3.3	12.3	5.9	3.7
	2	3.4	13.6	4.6	−1.8
RP 69012	0.01	14.7		−6.8	
	0.175	2.0	10.3	−2.7	5.7
	0.750	4.3	11.8	2.0	1.7
RPR 100391	0.01	10.0		−6.8	
	0.175	2.1	8.3	−1.8	5.8
	0.750	3.9	8.9	−0.3	1.1

3.4. Precision

As shown in Table 2, intra-day precision of the plasma assay was assessed by using six replicates of spiked plasma at three concentrations: at 0.025, 0.5 and 2.0 mg l^{-1}, coefficients of variation (C.V.s) were between 1.6 and 7.4% for RP 57669, RP 54476 and RP 12536. At 0.010, 0.175 and 0.750 mg l^{-1}, C.V.s were between 2 and 14.7% for RP 69012 and RPR 100391.

Inter-day precision (Table 2) was assessed by assaying plasma samples spiked at two concentration levels (0.5 and 2.0 mg l^{-1} for RP 57669, RP 54476 and RP 12536, and 0.175 and 0.750 mg l^{-1} for RP 69012 and RPR 100391) over two months.

C.V.s were between 9% and 19.4% for RP 57669, RP 54476 and RP 12536 and between 8.3 and 11.8% for RP 69012 and RPR 100391.

3.5. Accuracy

The accuracy of the intra-day analysis was evaluated by using six replicates of spiked plasma at three concentrations (0.025, 0.5, 2.0 mg l^{-1} for RP 57669, RP 54476 and RP 12536, 0.010, 0.175 and 0.750 mg l^{-1} for RP 69012 and RPR 100391; Table 2). The differences (Diff.%) between mean measured and nominal concentrations were calculated as follows: (mean conc.−nominal conc.)/nominal conc.×100.

The intra-day Diff% were between −7.4% and 0.09% (RP 57669), −14.4% and 12.3% (RP 54476), 4.6% and 5.9% (RP 12536), −6.8% and 2.0% (RP 69012) and −6.8% and −0.3% (RPR 100391).

Inter-day accuracy (Table 2) was assessed by assaying plasma samples spiked at two concentrations (0.5 and 2.0 mg l^{-1} for RP 57669, RP 54476 and RP 12536, and 0.175 and 0.750 mg l^{-1} for RP 69012 and RPR 100391) over two months under the same storage conditions.

The inter-day Diff% were between 2.0% and 2.5% (RP 57669), −10.1% and 5.1% (RP 54476), −1.8% and 3.7% (RP 12536), 1.7% and 5.7% (RP 69012) and 1.1% and 5.8% (RPR 100391).

3.6. Limit of quantitation

The limit of quantitation was determined at the concentration for which the C.V. was not more than

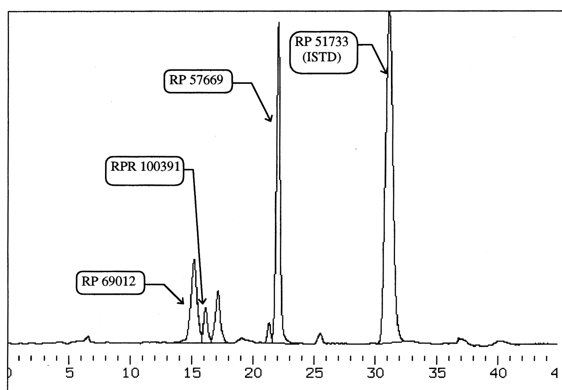

Fig. 6. Chromatogram obtained by fluorimetric detection of an extracted plasma from a subject at 55 min following 1-h i.v. infusion of 7.5 mg of quinupristin/dalfopristin.

15%. The limits of quantitation were 0.025 mg l^{-1} (C.V.=3.7%) for RP 57669, 0.010 mg l^{-1} (C.V.= 14.7%) for RP 69012, 0.010 mg l^{-1} (C.V.=10.1%) for RPR 100391, 0.025 mg l^{-1} (C.V.=7.4%) for RP 54476 and 0.025 mg l^{-1} (C.V.=1.6%) for RP 12536.

3.7. Stability

RP 57669, RP 54476 and RP 12536 were stable in human plasma under acidic conditions (pH=5–6) for 1 year at −20°C. RP 69012 and RPR 100391 were stable for at least two months.

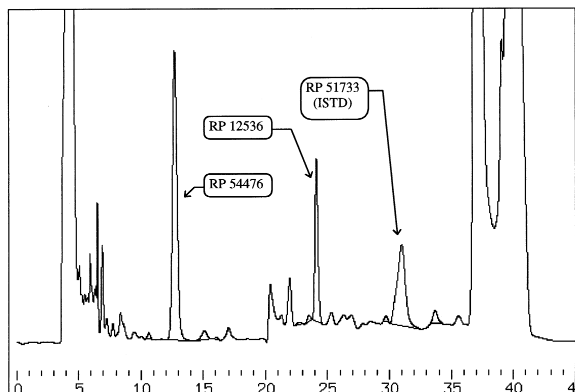

Fig. 7. Chromatogram obtained by UV detection of an extracted plasma from a subject at 55 min following 1-h i.v. infusion of 7.5 mg of quinupristin/dalfopristin.

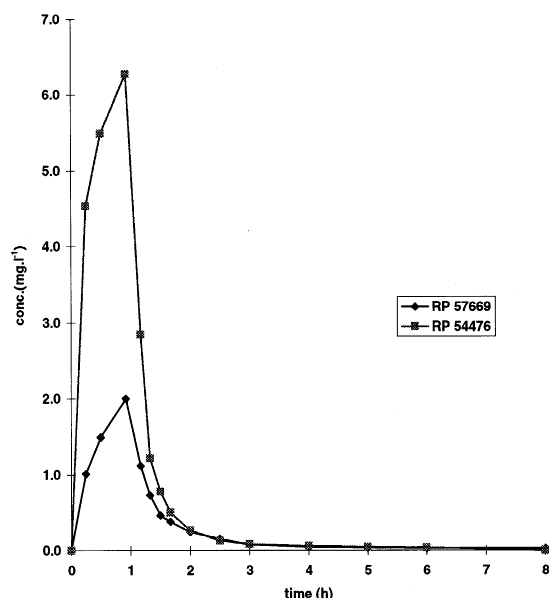

Fig. 8. Plasma concentration (mg l^{-1}) versus time (h) curve of parent drugs in a healthy subject after an 1-h i.v. infusion of 7.5 mg of quinupristin/dalfopristin.

3.8. Clinical applicability

This method was applied to pharmacokinetic studies of the drug in either volunteers or patients. Representative chromatograms of plasma samples taken 1 h after start of the intravenous (i.v.) infusion of 7.5 mg quinupristin–dalfopristin are shown in Figs. 6 and 7. The plasma concentration versus time curves are illustrated in Fig. 8 (parent drugs: RP 57669 and RP 54476) and Fig. 9 (main metabolites: RP 69012, RPR 100391 and RP 12536).

4. Conclusions

A specific, accurate and precise HPLC method for the simultaneous measurement of the concentration of quinupristin, dalfopristin and three metabolites of these compounds in human plasma was described. This method requires acidification of blood samples prior to extraction, two detection modes (UV and fluorescence detection), and a step gradient, where each analyte was eluted during an isocratic interval. Although being complex, the method is fully automated and allows on-line determination using

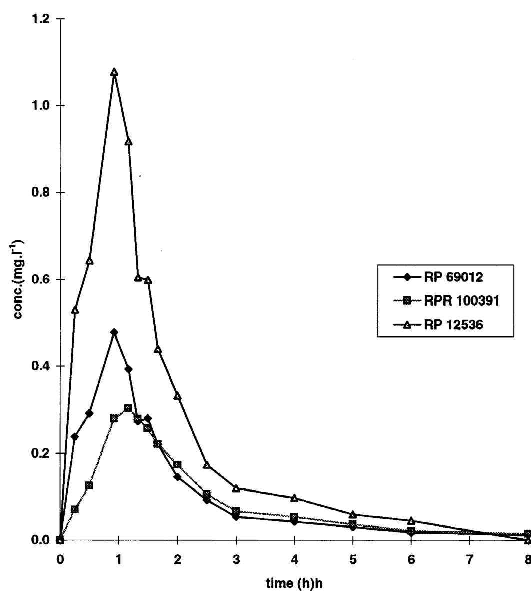

Fig. 9. Plasma concentration (mg l^{-1}) versus time (h) curve of main metabolites in a healthy subject after an 1-h i.v. infusion of 7.5 mg of quinupristin/dalfopristin.

acidified plasma samples. Furthermore the method is suitable for human pharmacokinetic studies.

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