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Determination of ritonavir, a new HIV protease inhibitor, in biological samples using reversed-phase high-performance liquid chromatography

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Abstract

A simple, accurate and precise high-performance liquid chromatographic method has been developed for measurement of ritonavir concentrations in human plasma. Ritonavir was partitioned from the plasma using liquid–liquid extraction with a mixture of ethyl acetate and hexane at neutral pH, with an average recovery >80%. Following two sequential washings of the reconstituted sample with hexane, chromatographic separation was accomplished on a C_{18} analytical column with a mobile phase containing acetonitrile, methanol and 0.01 *M* tetramethylammonium perchlorate in 0.1% aqueous trifluoroacetic acid (40:5:55, v/v) with low wavelength UV detection at 205 nm. Standard curves were linear (r^2>0.9998) over the concentration range 0.01–15 μg/ml with both inter- and intra-day coefficients of variation typically less than 5%. The stability of ritonavir in plasma was excellent, with no evidence of degradation after 5 days at room temperature or after 6 months in a freezer. Decontamination procedures for HIV-positive plasma samples showed 5.6 and 10.2% degradation following heating to 60°C for 30 or 60 min, respectively.

Keywords: Ritonavir

1. Introduction

Ritonavir (Norvir, ABT-538, (5*S*, 8*S*, 10*S*, 11*S*)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3, 6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester) (Fig. 1) is a peptidomimetic inhibitor of HIV protease with a unique combination of potency, selectivity and oral bioavailability [1]. Ritonavir exhibits potent in vitro activity against laboratory and clinical strains of HIV-1 [50% effective concentration (EC_{50})=0.022–0.13 μ*M*] and HIV-2 (EC_{50}=0.16 μ*M*). In the absence of reliable animal models for AIDS, the simultaneous optimization of both in vitro anti-HIV activity and pharmacokinetic behavior of antiviral agents provides the most reliable measure of potential activity in vivo. A pharmacokinetic profile which maintains systemic levels sufficient to completely block viral replication in vivo may be particularly important to prevent or delay the onset of resistance. In early trials in AIDS patients, orally administered ritonavir has produced profound declines in plasma viral RNA with con-

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comitant increases in CD4 cells [2,3]. Herein we describe the basic analytical method used for the determination of ritonavir concentrations in plasma in both animal and human pharmacokinetic and efficacy studies.

2. Experimental

2.1. Chemicals and reagents

Ritonavir, the internal standard (I.S., A-86093, (5*S*, 8*S*, 10*S*, 11*S*)-9-hydroxy-2-cyclopropyl-5-(1-methylethyl) - 1 - [2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl) - 2, 4, 7, 12 - tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester), the metabolites [A-98493 (M1), A-136478 (M2) and A-155679 (M11)] [4], and the ritonavir diastereomers [*SSRS* (A-98633), *SRSS* (A-98658), *SRRS* (A-98843) and *RSSS* (A-117673) (stereochemistry at chiral centers 5, 8, 10 and 11, respectively)] were synthesized at Abbott Laboratories (Abbott Park, IL, USA, Figs. 1 and 2). Tetramethylammonium perchlorate was purchased from Sigma (St. Louis, MO, USA) and trifluoroacetic acid was purchased from EM Science (Gibbstown, NJ, USA). All chemicals were reagent grade and were used as received without further purification. All solvents (methanol, acetonitrile, hexane, ethyl acetate) were of HPLC grade. The water used in the preparation of reagent solutions and mobile phase was purified with a Milli-Q Water System (Millipore, Bedford, MA, USA).

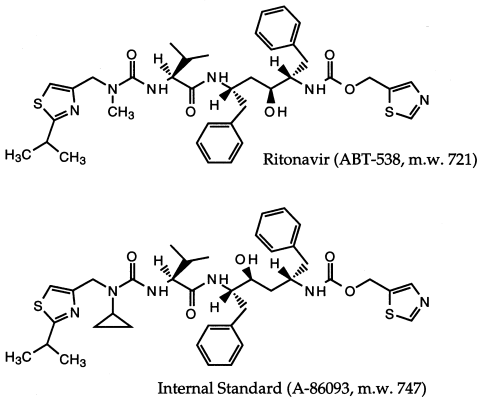

Fig. 1. Structures of ritonavir and the internal standard.

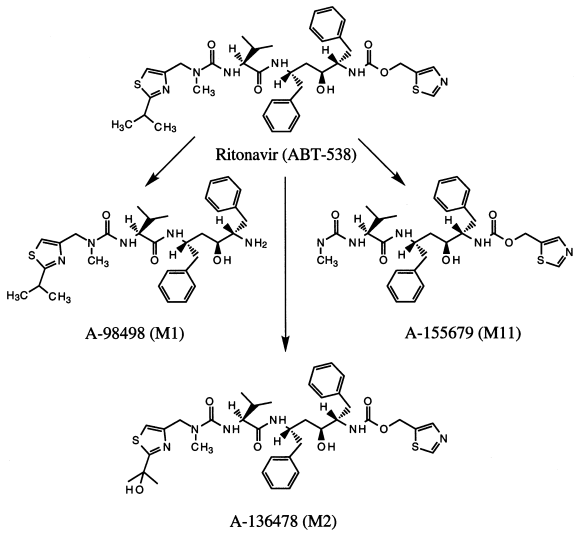

Fig. 2. Chemical structures of the primary ritonavir metabolites M1, M2 and M11 [4].

2.2. Chromatographic conditions

The HPLC system consisted of two Spectroflow Model 400 dual piston isocratic pumps (Applied Biosystems (ABI), Foster City, CA, USA), a Spectroflow Model 490 dynamic mixer (ABI), a Model 785A or 783A programmable absorbance detector (ABI) and a Model 655A-40 autosampler (Hitachi Instruments, Tokyo, Japan). The detector was operated at a wavelength of 205 nm. Chromatographic separation was accomplished on a ODS-AQ column (5 cm×4.0 mm I.D., 3 μm particle size, YMC, Wilmington, NC, USA); similar separation could be achieved on a 5 cm×4.6 mm 3 μm ODS-2 column obtained from Regis Technologies (Little Champ, Morton Grove, IL, USA). The mobile phase contained a mixture of acetonitrile, methanol and 0.01 *M* tetramethylammonium perchlorate in 0.1% aqueous trifluoroacetic acid (40:5:55, v/v), at a constant flow-rate of 1.0 ml/min at ambient temperature. The detector output was digitized by a Beckman Chromlink A/D converter (Beckman Instruments, Allen-

dale, NJ, USA) and processed using a VAX PeakPro system (Beckman Instruments).

2.3. Standard preparation

The initial stock solutions of ritonavir were prepared in absolute ethanol at a concentration of ~500 μg/ml. Intermediate dilutions of the stock standard were prepared in methanol–water (1:1, v/v) at concentrations 10-fold higher than the desired plasma concentrations. Each of the intermediate standards were diluted 10-fold with blank plasma to provide standards over the concentration range 0.01–15.0 μg/ml.

A stock solution of the internal standard, A-86093, was similarly prepared in ethanol at a concentration of ~500 μg/ml. The stock solution was diluted with methanol–water (3:7, v/v) to produce the working internal standard solution at concentration in the midrange of the standard curve. Both ritonavir and the I.S. stock solutions in ethanol were stored at 4°C. The solutions were stable for at least 3 months under these conditions.

2.4. Sample preparation

A 0.5-ml aliquot of plasma was combined with an equal volume of working internal standard solution and 5-ml of ethyl acetate–hexane (9:1, v/v) in a 15-ml glass culture tube. The samples were vortexed vigorously for 15 s followed by centrifugation at ~1800 *g* for 10 min (2500 rpm, 4°C). The organic layer (~4.5 ml) was transferred to a glass conical centrifuge tube and evaporated to dryness with a gentle stream of dry air over low heat (~35°C). The samples were reconstituted by vortexing for 20 s with 0.3 ml of solution containing acetonitrile–methanol–0.01 *M* tetramethylammonium perchlorate in 0.1% aqueous trifluoroacetic acid (17:5:78, v/v). Each reconstituted sample was washed twice with 3 ml of hexane (vortexed ~2 s), to remove co-extracted contaminants. Following each hexane wash, the samples were centrifuged as described above. The hexane layer was removed by aspiration. The aqueous layer was transferred to autosampler vials with glass micro inserts for HPLC analysis. A 100-μl injection volume was used throughout the analytical validation and batch analysis.

2.5. Calibration and calculation procedures

Daily standard curves were constructed using the ratios of the observed peak area of ritonavir and the internal standard. The unknown concentrations were computed from the unweighted linear regression equation of the peak-area ratio against concentration for the calibration curve.

The inter- and intra-day accuracy and precision of the assay method were assessed at ritonavir concentrations in the range of 0.2–13.7 μg/ml. Three replicates were assayed for intra-day accuracy and thirty samples were used for inter-day variability. The relative error (accuracy) is expressed as the percentage deviation (±) from the theoretical concentration of the sample. The precision is expressed in terms of %C.V.

2.6. Stability

The stability of ritonavir in human plasma was evaluated at low (1.71 μg/ml), medium (5.14 μg/ml) and high (13.71 μg/ml) concentrations. Triplicate samples were analyzed on a daily basis following room temperature storage (~22°C) for five consecutive days. The day of the initiation of the stability trial was designated Day 0.

The stability of ritonavir in human plasma at the end of three freeze–thaw cycles was also evaluated at nominal concentrations of 0.15 and 12.0 μg/ml. To evaluate the stability of the parent drug under conditions suggested for the decontamination of HIV-containing plasma samples, human plasma samples containing ritonavir concentrations of 0.30, 3.63 and 8.47 μg/ml were heated at 60°C for both 30 and 60 min prior to analysis.

A series of ritonavir plasma samples, prepared over the concentration range 0.24–15.80 μg/ml, were placed in either polypropylene cryogenic tubes or in glass scintillation vials. The samples were frozen (−20°C) for 24 h and analyzed in duplicate against a set of freshly prepared (non-frozen) plasma standards. Quality control samples at nominal concentrations of 0.15, 7.50 and 12.0 μg/ml were analyzed at selected time points over a 6-month interval to provide an assessment of long term freezer stability (−80°C).

3. Results and discussion

3.1. Chromatography

A representative chromatogram from human plasma samples processed using the analytical method described above is provided in Fig. 3. Ritonavir elutes with a retention time of 7–9 min; the internal standard elutes 3–4 min later. Drug-free plasma samples obtained from normal subjects were devoid of interference near the retention times of both ritonavir and the internal standard. The ritonavir metabolites M1, M2 and M11 (Fig. 2) were well resolved from both ritonavir and the I.S. under these chromatographic conditions (Fig. 4). The HPLC conditions also provided baseline resolution between the *SSRS*, *SRSS*, *SRRS* and *RSSS* diastereomers of ritonavir (*SSSS*, Fig. 5). These representative chromatograms support the specificity of the analytical procedure.

A substantial collection of late-eluting contaminants was noted in the reconstituted plasma extracts during the initial method development (Fig. 6). The protonation of ritonavir and the internal standard with the acid-based mobile phase during reconstitution followed by two sequential washes with hexane removed a large fraction of these contaminants, greatly improving the efficiency of the method through a reduction in the chromatographic run time per sample.

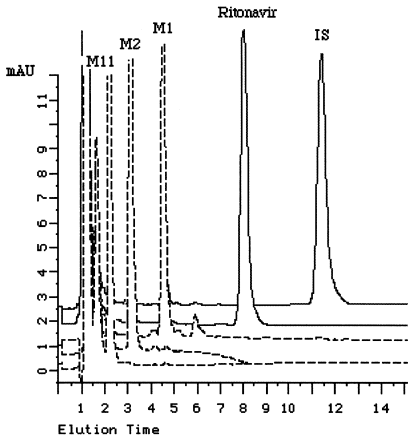

Fig. 4. Elution behavior of ritonavir metabolites M1, M2 and M11 under the chromatographic conditions utilized for the quantitation of ritonavir in plasma samples.

3.2. Calibration curve and limit of quantitation

The analysis of ritonavir in plasma exhibited excellent linearity (r^2>0.9998) over the 0.01–13.7 μg/ml concentration range. Regression intercepts for the calibration curves were generally very small and were not statistically significant compared to zero. A triplicate series of standards was typically analyzed

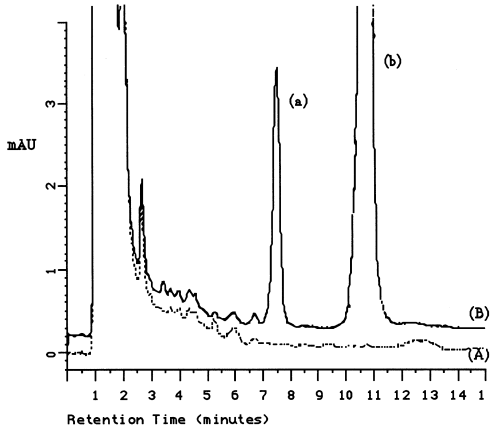

Fig. 3. Representative chromatograms from the quantitation of ritonavir (a) in human plasma; peak (b) is the internal standard. Trace (A) is a plasma blank; trace (B) contains ~0.1 μg/ml ritonavir (a) and the internal standard (b).

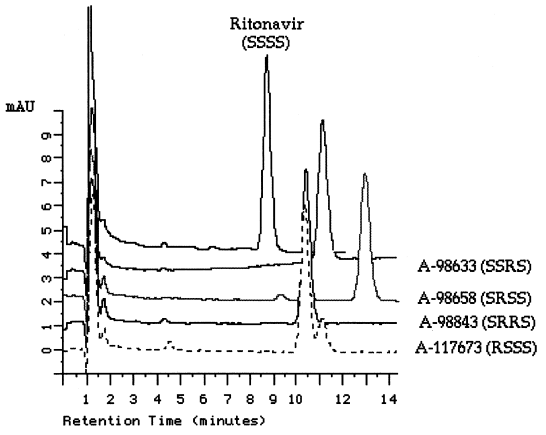

Fig. 5. Elution behavior of the ritonavir diastereomers under the chromatographic conditions utilized for the quantitation of ritonavir in plasma samples.

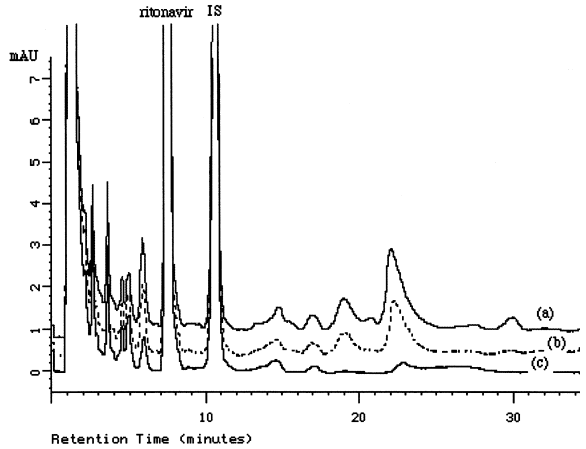

Fig. 6. Plasma extract containing ritonavir and the internal standard subjected to one (b) or two (c) hexane wash steps following reconstitution in mobile phase. Trace (a) was obtained from a non-washed sample.

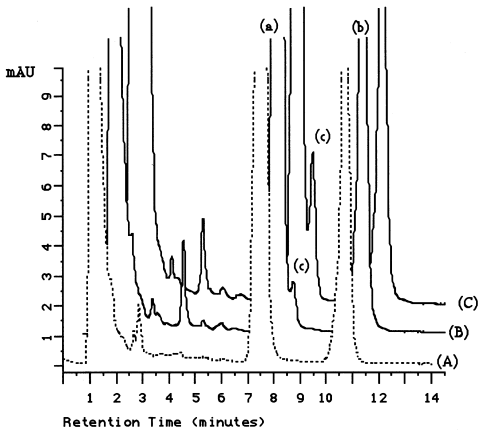

Fig. 7. Effect of pH on the stability of ritonavir during extraction from plasma. Sample (A) was extracted at neutral pH, sample (B) contained sodium bicarbonate and sample (C) contained sodium carbonate. Chromatographic peak (a) is ritonavir, peak (b) is the internal standard and peak (c) is an unidentified decomposition product of ritonavir.

together with the study samples. These daily calibration curves were used for calibration and calculation purposes. A series of spiked human plasma samples prepared at six separate ritonavir concentrations between 0.005 and 0.115 μg/ml, analyzed in triplicate, evaluated the lower limit of quantitation. The results indicated that the assay had acceptable precision (<20% C.V.) and accuracy (relative error ≤15%) to a lower limit of quantitation of 0.012 μg/ml (Table 1) with a 0.5-ml sample volume. Under the recommended dosage regimen of 600 mg every 12 h, the mean C_{min} is over 2 μg/ml. Thus the method has sufficient sensitivity for both clinical monitoring and pharmacokinetic purposes.

3.3. Recovery

The recovery of ritonavir and the I.S. was evaluated under both neutral and alkaline extraction conditions. While the recovery of both compounds was slightly higher under alkaline conditions (data not shown), a pH-dependent degradation was noted in samples extracted following addition of either sodium bicarbonate or sodium carbonate to the plasma (Fig. 7). The extraction recovery of ritonavir from spiked plasma samples at neutral pH, as evaluated at both a high (12.0 μg/ml) and low (0.15 μg/ml) concentration, averaged 81.6% and 86.9%, respectively. The recovery of the internal standard, as evaluated at a concentration of 7.5 μg/ml, was 76.3%.

3.4. Accuracy and precision

The within-run accuracy and precision of the method were estimated from the back-calculated standard concentrations (Table 2). The overall mean

Table 1
Limit of quantitation assessment for the measurement of ritonavir in human plasma

Theory (μg/ml)	0.115	0.092	0.046	0.023	0.012	0.005
Found (μg/ml)	0.117	0.096	0.047	0.024	0.014	0.003
S.D.	0.005	0.006	0.003	0.000	0.0005	0.003
C.V. (%)	4.3	5.8	6.9	0.0	3.3	121.5
Relative error (%)	1.6	3.8	1.2	5.6	14.5	−48.4

Table 2
Intra- and inter-day accuracy and precision for the determination of ritonavir in human plasma

Intra-day							
Theory (μg/ml)	12.10	8.467	6.048	3.629	1.210	0.605	0.302
Found (μg/ml)	12.00	8.660	5.966	3.633	1.228	0.573	0.296
S.D.	0.31	0.107	0.051	0.012	0.007	0.048	0.002
C.V. (%)	2.62	1.23	0.85	0.32	0.59	3.13	0.81
Relative error (%)	−0.79	2.28	−1.35	0.11	1.54	5.34	1.98
Minimum	11.66	8.563	5.981	3.622	1.221	0.552	0.294
Maximum	12.28	8.774	6.008	3.645	1.236	0.587	0.299
n	3	3	3	3	3	3	3
Inter-day							
Theory (μg/ml)	13.71	8.570	6.856	5.142	3.428	1.714	0.206
Found (μg/ml)	13.74	8.479	6.860	5.009	3.445	1.665	0.210
S.D.	0.46	0.394	0.352	0.227	0.155	0.074	0.010
C.V. (%)	3.31	4.64	5.13	4.53	4.51	4.45	4.65
Relative error (%)	0.21	−1.06	0.06	−2.58	0.49	−2.88	2.25
Minimum	12.76	7.765	6.253	4.423	3.122	1.525	0.231
Maximum	14.52	9.470	7.478	5.440	3.834	1.873	0.191
n	29[a]	30	30	30	30	30	23[b]

[a] One chromatographic failure.
[b] Outliers with ≥15% relative error excluded.

precision, as defined by the coefficient of variation (C.V.), ranged from 0.32% to 3.13% for plasma samples with relative errors ranging from 0.11% to 5.34% from triplicate standards analyzed within the same day. Inter-day variability, as estimated from the triplicate analysis of seven samples on ten separate days (Table 2), was low, with C.V. values ranging from 3.31% to 5.13%, and with relative errors ranging from 0.06% to 2.88% over the concentration range 0.206–13.71 μg/ml. These results indicated that this assay was reliable and reproducible.

3.5. Ritonavir stability

The stability of ritonavir in human plasma subjected to three sequential freeze (−80°C)–thaw (room temperature) cycles was evaluated at 0.15 and 12.0 μg/ml. Analyzed at the end of the three freeze–thaw cycles, the ritonavir concentrations averaged 101% and 92.3% of the baseline concentration (data not shown).

The data derived from ritonavir human plasma samples, prepared at low, medium and high concentrations in the 1–15 μg/ml concentration range, and analyzed following 0, 1, 3 and 5 days storage at room temperature are provided in Table 3. There was no evidence of degradation of ritonavir in plasma under these conditions. The change in the concentration on Day 5 compared to Day 0 averaged −13.7, +3.3 and −0.3% for the low, medium and high concentrations, respectively. Collectively, these data indicate that ritonavir is quite stable in plasma at room temperature for at least 5 days.

To evaluate the stability of ritonavir in plasma under conditions utilized for decontamination, samples at 0.30, 3.63 and 8.47 μg/ml were placed in a constant temperature oven at 60°C for either a 30- or 60-min time interval. Slight degradation (average −5.6%) was noted following 30 min at the elevated temperature. The average degradation doubled to 10.3% following 60 min at 60°C.

Excellent recovery was found for ritonavir plasma samples frozen (−20°C) in both glass and polypropylene storage containers (Table 4). At each of

Table 3
Stability of ritonavir in human plasma at room temperature

	Plasma concentration (μg/ml) (% change)[a]		
	Low	Medium	High
Day 0	1.805	5.564	15.17
Day 1	1.773 (−1.8)	5.753 (3.4)	15.49 (2.1)
Day 3	1.571 (−13.0)	5.755 (3.4)	15.40 (1.6)
Day 5	1.558 (−13.7)	5.746 (3.3)	15.12 (−0.3)

[a] Percent change in ritonavir concentration on days 1, 3, 5 compared to day 0.

Table 4
Recovery of ritonavir from plasma samples frozen (−20°C) in glass or polypropylene storage containers

Theory (μg/ml)	Frozen in glass		Frozen in polypropylene	
	(μg/ml)	RE(%)	(μg/ml)	RE(%)
0.000	0.000	−	0.000	−
0.237	0.229	−3.4	0.229	−3.4
0.553	0.533	−3.6	0.563	+1.8
1.106	1.088	−1.6	1.105	−0.1
1.580	1.586	+0.4	1.524	−3.6
4.741	4.528	−4.5	4.425	−6.7
7.902	7.694	−2.6	7.981	+1.0
11.06	10.94	−1.1	11.20	+1.3

RE=relative error expressed as a percent.

seven separate concentrations, the concentration recovered from the frozen samples was generally no more than 4% (±) of the concentration obtained from freshly prepared plasma samples over the 0.237–11.06 μg/ml concentration range.

Quality control samples analyzed over a 6-month elapsed time period at nominal concentrations of 0.15, 7.50 and 12.0 μg/ml were characterized by C.V. values of 6.64%, 7.29% and 5.54% (n=35, 50 and 36), with 3.8%, −11.3% and −10.3% relative error at the end of the storage period. This data demonstrates acceptable freezer stability for a 6-month time period (−80°C).

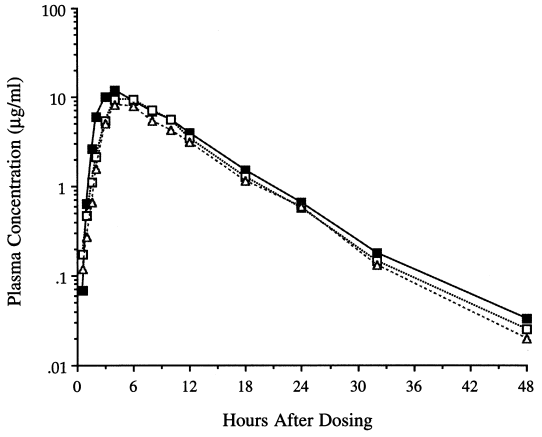

Fig. 8. Mean (n=11) ritonavir plasma concentration–time profiles derived from a 500-mg oral dose of three experimental liquid formulations in healthy subjects.

3.6. Assay application

The described analytical method was used to determine the plasma concentrations of ritonavir following a 500-mg oral dose of three experimental liquid formulations in healthy subjects (Fig. 8). Peak plasma concentrations averaging 9.8–12.4 μg/ml were obtained ~5 h after dosing; ritonavir concentrations remained above the limit of quantitation 48-h after dosing. Concentrations of ritonavir declined with a plasma elimination half-life of 5 h.

4. Conclusions

A highly specific, sensitive and rapid method has been developed for the quantitation of ritonavir in human plasma samples. Without modification, the method has also been applied to the measurement of parent drug concentrations in rat, rabbit, mouse, dog and monkey plasma samples derived from pre-clinical pharmacokinetic studies and from homogenates of thymus, mesenteric lymph nodes, cervical lymph nodes and brain obtained following an oral dose in rat [1].

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