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LC separation and induced fluorometric detection of rivastatin in blood plasma*

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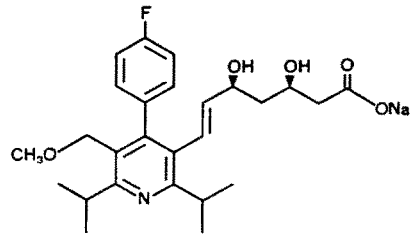
Abstract: An LC procedure suitable for quantitative analysis of pg ml^{-1} concentrations of the HMG-CoA reductase inhibitor rivastatin in blood plasma was developed. The procedure involves an extraction step, chromatography on an ODS column, and fluorometric detection of a post-column photolytic decomposition product that was isolated and identified. The achieved quantitation limit (25 pg ml^{-1}) facilitated analysis of relatively low rivastatin concentrations in plasma that were observed after 100–300 μg oral doses of rivastatin. At 25 pg ml^{-1} concentration the RSD ranged from 3.6 to 13.5% and mean deviation from the nominal value was 8.0%; at 8 ng ml^{-1} the RSD range was 0.7–3.6% while the mean deviation was −1.8%. The concentrations obtained with the LC procedure were compared to the concentrations obtained with a specific but less sensitive capillary GC method and a radioimmunoassay (RIA) procedure. Concentrations obtained with the HPLC and GC procedures agreed within experimental error; the RIA concentrations were about 30% higher.

Keywords: *Rivastatin; HPLC; fluorescence induction; blood plasma assay.*

Introduction

Enzymatic reduction of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) to mevalonate is one of the rate limiting steps of cholesterol biosynthesis [1, 2]. Three different drugs that inhibit biosynthesis of mevalonate by HMG-CoA reductase and subsequently biosynthesis of cholesterol, were approved by FDA as cholesterol lowering agents. Two of the drugs, lovastatin and simvastatin, are lactone prodrugs that are converted to the active free form after oral administration. The more recently approved pravastatin unlike lovastatin and simvastatin is administered in the active free-acid form.

Figure 1 depicts the chemical structure of rivastatin (Bay w 6228). Rivastatin is a synthetic, enantiomerically pure HMG-CoA reductase inhibitor that is about 100 times more active than lovastatin [3–5] and like pravastatin is administered in the active free-acid form. The relatively high HMG-CoA reductase inhibitory activity of rivastatin facilitated rather low (100–300 μg) oral doses which yielded very low concentrations (0.05–5 ng ml^{-1}) of rivastatin in blood plasma.



(+)-3R,5S-Sodium-erythro-(E)-7-[4-(4-fluorophenyl)-2,6-diisopropyl-5-methoxy-methyl-pyrid-3-yl]-3,5 dihydroxy-hept-6-enoate

Figure 1
The chemical structure of rivastatin.

Consequently, a sensitive procedure for analysis of rivastatin was necessary to obtain meaningful pharmacokinetic data.

A semi-specific radioimmunoassay [6] and a specific capillary GC procedure have already been developed for analysis of rivastatin in body fluids [7]. However, the capillary GC procedure involves two derivatization steps prior to chromatography and has a 0.5 ng ml^{-1} quantitation limit. Since the observed concentrations of rivastatin in blood plasma can be significantly lower than 0.5 ng ml^{-1} even a few hours after 100 and 200 μg doses of rivastatin,

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an alternative HPLC procedure was developed.

The LC procedure involves chromatographic separation of underivatized rivastatin from constituents of plasma extract on a reversed-phase ODS column followed by a post-column UV induction of fluorescence. The quantitation limit of the induced fluorometric signal is 25 pg ml^{-1}; fluorometric quantitation limit based on native fluorescence of rivastatin is about 75 times less sensitive.

Experimental

Materials and reagents

Analytical grade potassium dihydrogen phosphate and HPLC grade hexane and 85% O-phosphoric acid were obtained from Fischer Scientific (Springfield, NJ). HPLC grade acetone, acetonitrile, and methanol and Resi-analysed reagent grade ether were obtained from J.T. Baker (Phillipsburg, NJ). Milli-Q Reagent water system (Millipore, Bedford, MA) was used for purification of deionized water. Human plasma was obtained from Biological Specialty (Landsdale, PA). Rivastatin, internal standard (analogue of rivastatin) and demethylated metabolite of rivastatin were obtained from Institute of Clinical Pharmacology (5600 Wuppertal 1, Germany).

Stock solutions of rivastatin and internal standard were prepared in acetone at 0.1 mg ml^{-1} concentration and diluted with water (1:50, v/v) prior to addition to plasma. The concentration of rivastatin in plasma ranged from 0.025 to 8.0 ng ml^{-1}. Stock solution of internal standard was diluted with water to yield 8 ng ml^{-1} concentration.

Sample preparation

Aliquots (1 ml) of plasma reference standard, quality control, and subject samples were vortexed with 0.5 ml aliquot of aqueous internal standard solution and 1 ml aliquot of 1 M, pH 5.5 phosphate buffer solution. The above mixture was extracted for 20 min with 4 ml of ether–hexane (3:1, v/v). After centrifugation at 2800 rpm for 10 min, 3.5 ml of the organic phase was withdrawn and evaporated at 50°C under nitrogen in a clean glass test tube. The residue was reconstituted in 0.3 ml of the aqueous component of chromatographic solvent described below. The reconstituted solution was transferred into a tapered auto-sampler vial.

Chromatography

Aliquots (80–90 μl) of the reconstituted solution were injected onto a 25 cm × 4.6 mm i.d. column packed with 5 μm spherical C_{18} support. Extrasil column obtained from Phenomenex (Torrance, CA) or Inertsil column obtained from Metachem (Redondo Beach, CA) was used for this purpose. The column was eluted with acetonitrile–methanol–potassium dihydrogen phosphate (pH 2.5, 0.01 M) (10:4:10, v/v/v) chromatographic solvent at 1.4–1.6 ml min^{-1} flow rate. The column temperature was maintained at 54–55°C.

Detection and quantitation

Column eluent was exposed to UV light emitted by a low pressure mercury lamp. A 10 m, 0.25 mm i.d. knitted coil mounted in a post-column reactor obtained from Aura Industries (Staten Island, NY) was used for this purpose. A Waters Associates Model 470 (Milford, MA) scanning fluorescence detector or Hitachi Model F-1050 (Danbury, CT) fluorescence detector was used to measure the induced fluorescence at 290–295 nm excitation and 390 nm emission wavelengths. Detector output was recorded on a Spectra-Physics Model SP4270 (San Jose, CA) chromatographic data module. Peak height ratios were used for quantitation of rivastatin concentrations.

Results and Discussion

Chromatographic selectivity and efficiency

Figures 2 and 3 illustrate representative chromatograms obtained with extracts of blank plasma, plasma containing known concentrations of rivastatin and internal standard and plasma collected from subjects enrolled in clinical studies. It is apparent that the chromatographic system separated rivastatin from internal standard, demethylated metabolite, and unrelated constituents of plasma extracts. Although minor peaks of endogenous compounds were observed in the vicinity of rivastatin these peaks were smaller than the peak observed with the lowest quantitative concentration of rivastatin. The shoulder of a minor peak that overlaps partially with the internal standard contributed less than 0.5% to the internal standard peak height and the observed accuracy and precision of the assay did not require any compensation for this overlap.

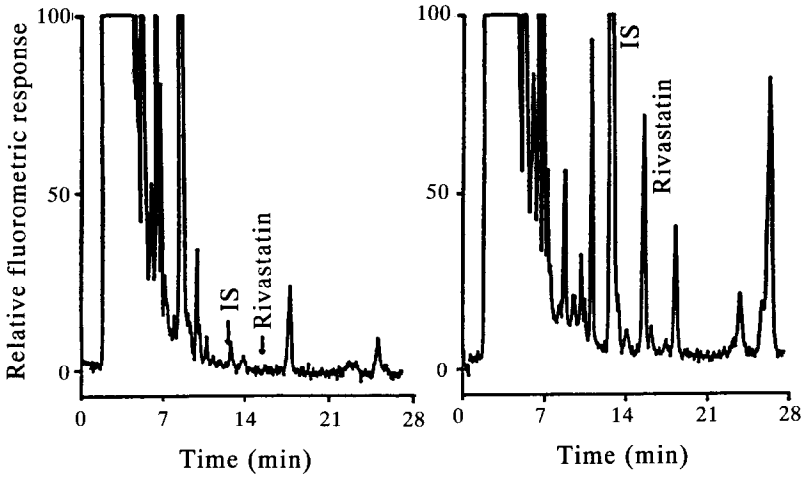

Figure 2
HPLC chromatograms of a blank of a plasma extract (left chromatogram) and an extract of plasma containing 0.3 ng ml^{-1} of rivastatin and 4 ng ml^{-1} of internal standard (right chromatogram).

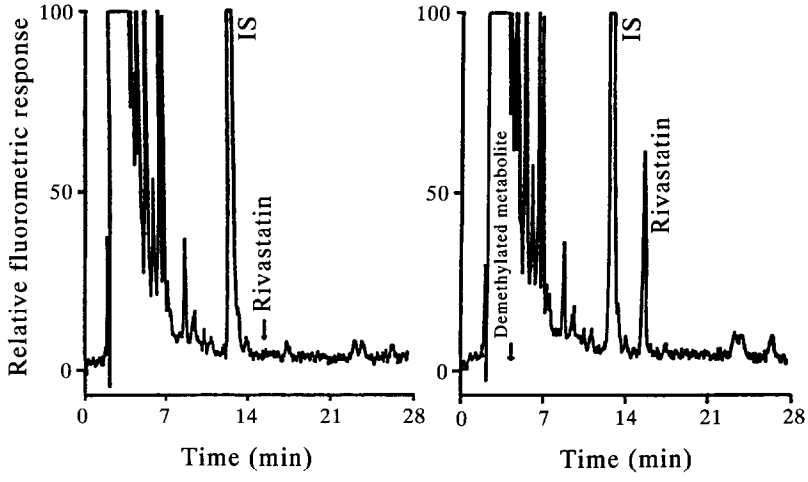

Figure 3
HPLC chromatograms of a pre-dose clinical plasma extract containing 4 ng ml^{-1} of internal standard (left chromatogram) and a post-dose clinical plasma extract containing 0.25 ng ml^{-1} of rivastatin and 4 ng ml^{-1} of internal standard (right chromatogram).

Relative chromatographic efficiency of several different reversed-phase C_{18} columns was investigated. The theoretical plates of the selected C_{18} column ranged from 9000 to 14,000. Although chromatographic efficiency increased from 14,000 plates at 1.5 ml min^{-1} to 17,000 plates at 1.0 ml min^{-1}, in order to decrease chromatographic run time a 1.4–1.6 ml min^{-1} flow rate was selected.

Detection and quantitation limit

Rivastatin is fluorescent. However, the native fluorescence of rivastatin is about 75 times lower than the fluorescence of the UV photolytic decomposition product. Figure 4 contrasts chromatograms observed before and after UV induction. The observed amplification of the fluorometric signal after UV induction is due to the photolytic decomposition product that was isolated by chromatography and identified by mass spectroscopy. The decomposition product was also synthesized and its structure was confirmed by NMR, MS and chromatographic analysis. Figure 5 illustrates the structure of this decomposition product. This structure is consistent with the observation that the decomposition product is considerably less polar than rivastatin because it is retained much longer on a reversed-phase column.

The intense fluorescence of this product may be attributed to reduced interaction of this

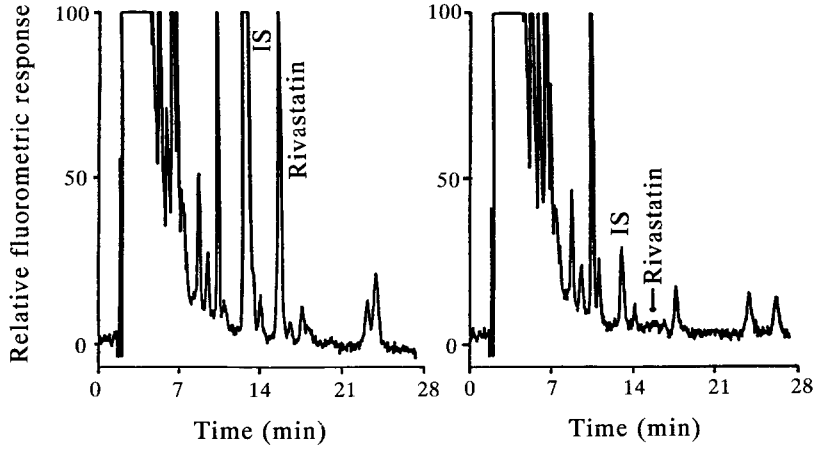

Figure 4
HPLC chromatograms of a pre-dose clinical plasma extract containing 0.5 ng ml^{-1} of rivastatin and 4 ng ml^{-1} of internal standard obtained with UV photoreactor on (left chromatogram) and off (right chromatogram).

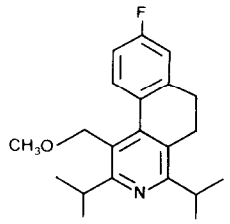

Figure 5
The structure of the photolytic decomposition product of rivastatin.

hydrophobic molecule with relatively more polar chromatographic solvent molecules and this reduced interaction suppresses external quenching of the excited singlet state. In contrast, the polar side chain of the rivastatin molecule enhances solvent interaction and external quenching of rivastatin fluorescence. However, internal rotational and vibrational effects of the rivastatin molecule may also facilitate internal quenching of rivastatin fluorescence. The internal quenching effect of rotation accounts for the observed lack of phenolphthalein fluorescence and intense fluorescence of fluorescein which has a similar structure to phenolphthalein except for more restricted rotation [8].

The established lower quantitation limit (LQL) of the production was 25 pg ml^{-1}. At this LQL the signal-to-noise ratio of rivastatin was about 5, the RSD ranged from 3.6 to 13.5% and mean deviation from the nominal value was about 8%.

Linearity of response

A linear relationship between response ratios and concentrations of reference standards was observed within the concentration range specified in the procedure (0.025–8.0 ng ml^{-1}). The following equation defined the observed linear relationship: Resp. Ratio = 0.00476 + 0.2467 Conc. The observed correlation coefficient was 0.999. Since none of the plasma samples collected during clinical studies contained concentrations of rivastatin higher than 8.0 ng ml^{-1} it was not necessary to extend the concentration range beyond 8.0 ng ml^{-1} where non-linearity due to fluorescence quenching can be observed.

Recovery

Table 1 lists percentage recoveries of rivastatin at different concentrations and internal standard at the specified concentration after liquid–liquid extraction step. The listed percentage recoveries are corrected for known losses due to partial (90%) withdrawal of the organic phase. The observed mean recoveries of rivastatin and internal standard were 88% for both compounds and percentage recovery did not vary significantly with concentration of rivastatin in plasma.

Accuracy and precision

Tables 2 and 3 present the intra-day and the inter-day accuracy and precision data that were obtained with plasma calibration standards and plasma quality control samples. The data presented in these tables indicate that the intra-day RSD ranged from 0.4 to 5.0% while mean deviation (bias) ranged from −4.5 to 6.0%. The inter-day precision and accuracy

Table 1
Recoveries of rivastatin and internal standard from human plasma

Theoretical conc. (ng ml^{-1})	Replicates*	Rivastatin mean recovery (%)	SD	RSD (%)	Internal standard mean recovery (%)	SD	RSD (%)
0.05	6	84	1.97	2.3	86	4.45	5.2
0.10	6	87	3.32	3.8	87	5.00	5.7
0.50	6	88	1.89	2.2	89	3.39	3.8
1.00	6	89	3.29	3.7	88	4.19	4.8
2.00	6	87	2.54	2.9	87	3.72	4.3
4.00	6	88	4.78	5.4	88	5.72	6.5
8.00	6	91	2.08	2.3	90	2.85	3.2

*Three sets of calibration standards containing duplicate standards analysed on separate days.

Table 2
Intra-assay* precision and bias of rivastatin in human plasma

Theoretical conc. (ng ml^{-1})	Replicates	Mean observed conc. (ng ml^{-1})	RSD (%)	Bias† mean deviation (%)
0.025	6	0.026	5.0	4.0
0.050	6	0.053	3.5	6.0
0.10	6	0.10	2.4	0.0
0.50	6	0.51	2.7	2.0
1.00	6	1.00	0.6	0.0
2.00	6	1.93	1.1	−3.5
4.00	6	3.85	0.4	−3.8
8.00	6	7.64	1.0	−4.5

*Value 0.9999, slope 0.2476.
†Defined as $\frac{[\text{Found}] - [\text{Theory}]}{[\text{Theory}]} \times 100$.

Table 3
Inter-assay precision and bias of rivastatin in human plasma

Theoretical conc. (ng ml^{-1})	Replicates*	Mean observed conc. (ng ml^{-1})	RSD (%)	Bias† mean deviation (%)
0.040	140	0.041	4.5	2.5
0.25	312	0.25	5.2	0.0
0.50	30	0.51	3.1	2.0
1.00	384	0.98	4.1	−2.0
2.00	23	2.03	1.9	1.5
3.00	264	2.95	3.9	−1.7
4.00	120	3.84	4.0	−4.0
8.00	23	7.87	2.4	−1.6

*Data obtained during seven clinical studies. At least three concentration levels were analysed in duplicate during each clinical assay run. Assay validation runs included three concentration levels in triplicate.
†Defined as $\frac{[\text{Found}] - [\text{Theory}]}{[\text{Theory}]} \times 100$.

was based on analysis of quality control plasma samples that were analysed concurrently with every set of clinical study samples. Since this procedure was applied to the analysis of over 6000 plasma samples that were collected during clinical studies, analysis of the inter-day precision and accuracy is based on a relatively large number of quality control samples. According to this analysis, all RSD and mean deviation values were below 10%.

Clinical pharmacology applications

The developed LC procedure was applied to analysis of plasma samples collected during five clinical pharmacology studies. Some of the pharmacokinetic data obtained with this procedure were already presented at the American Society for Clinical Pharmacology and Therapeutics Annual Meeting [7]. Results obtained with other clinical pharmacology studies were submitted for presentation at the

American Association of Pharmaceutical Scientists Meeting in Orlando, US, November 1993.

Figure 6 illustrates representative concentrations vs time data that were obtained with plasma samples collected during a multiple dose proportionality study. It is apparent that the LC procedure is sufficiently sensitive to measure concentrations of rivastatin in plasma 24 h after the dose. Pharmacokinetic analysis of the data obtained during this study will be published separately. Pharmacokinetic analysis of the data obtained during this study will be published separately. Figure 7 compares LC concentrations observed in a set of clinical study samples with the capillary GC and radioimmunoassay (RIA) concentrations. The consistently higher RIA results may be attributed to the semi-specific nature of the (RIA) and possible presence of a metabolite and/or endogenous compound that also yields RIA response. The apparent agreement between the GC and LC assay results suggests that both chromatographic procedures are specific.

Conclusions

A significant (75-fold) enhancement of rivastatin fluorescence was observed after post-column exposure of column eluent to UV light. The UV induced fluorescence yielded 25 pg ml^{-1} quantitation limit and may be attributed to the formation of an intensely fluorescent derivative. The HPLC procedure involving detection of induced fluorescence was found to be sufficiently accurate, precise, sensitive and specific for analysis of plasma samples collected during clinical pharmacology studies in which 100–400 μg oral doses of rivastatin were administered.

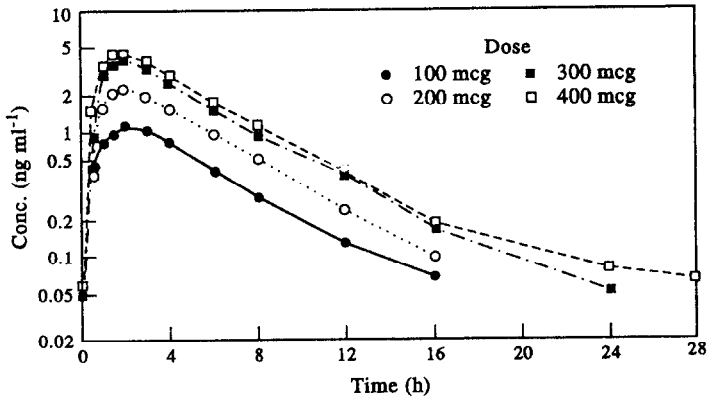

Figure 6
Steady state mean plasma concentrations of rivastatin after a single daily dose to healthy male volunteers for 7 days.

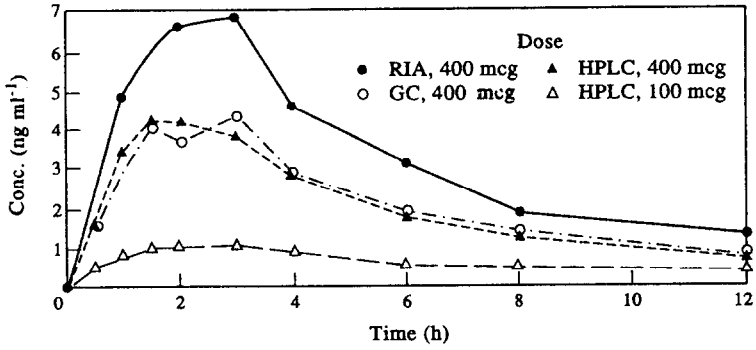

Figure 7
Comparison of RIA, HPLC and GC concentrations of rivastatin in plasma samples collected during rivastatin pharmacokinetic study.

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