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A Selected Ion Monitoring Method for Quantifying Simvastatin and Its Acid Form in Human Plasma, Using the Ferroceneboronate Derivative

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Simvastatin, a pro-drug lactone, forms the open carboxylic acid as a major metabolite that inhibits the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Simvastatin and the acid in plasma were quantified by a gas chromatography/mass spectrometry/selected ion monitoring (GC/MS/SIM) method. These drugs were separated by solid-phase extraction and independently converted into a 1,3-diol-type compound. This compound reacted with ferroceneboronic acid to yield the cyclic boronate that gave satisfactory mass spectra for GC/MS/SIM measurements. The spectrum was dominated by the molecular ion appearing as the base peak, thereby leading to a sensitive and selective assay. The calibration curves for simvastatin and the acid were linear in their concentration range of 0.1–10 ng ml^{-1}, where the values of coefficient of variation for both drugs were below 8%, except for the value of 11% for simvastatin at a concentration of 0.1 ng ml^{-1}. The quantification limit for both drugs was 0.1 ng ml^{-1} on the basis of a signal-to-noise ratio of 4:1.

INTRODUCTION

Simvastatin (SV), containing a lactone moiety as shown in Fig. 1, is being developed as a pro-drug as an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Although SV itself is pharmacologically inactive, the lactone is hydrolysed to form the carboxylic acid (**1a**) as a major metabolite that acts as an inhibitor of HMG-CoA reductase and thus of cholesterol synthesis.[1–4]

An enzyme inhibition assay to measure HMG-CoA reductase inhibitors such as Lovastatin (LV)[5] and SV[6] is fairly sensitive; however, it is not specific because it measures all of the inhibitors present. The high-performance liquid chromatography (HPLC) method reported in the literature[7] is selective, but not sensitive enough for determination of the levels of SV and acid **1a** in human plasma.

The present investigation was undertaken to examine whether an alternative method could be devised for a sensitive and selective analysis of SV and acid **1a** in human plasma. A gas chromatography/mass spectrometry/selected ion monitoring (GC/MS/SIM) method was employed, since it was expected to provide good sensitivity and selectivity. Ferroceneboronate **3a** was found to fulfil the requirements of the mass spectrometric evaluations; the derivative afforded a sharp and symmetrical GC peak and a mass spectrum in which the molecular ion predominated and appeared as the base peak, as in the case of various cyclic ferroceneboronates.[8,9]

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METHODS AND RESULTS

Materials

SV, LV and their acids (as the ammonium salts of **1a** and **1b**) were supplied by Merck Sharp and Dohme Research Laboratories (MSDRL): SV; (+)-(1*S*,3*R*,7*S*,8*S*,8a*R*)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2*R*,4*R*)-tetrahydro-4-hydroxy-6-oxo-2*H*-pyran-2-yl]ethyl]-1-naphthyl 2,2-dimethylbutanoate, and LV; (+)-(1*S*,3*R*,7*S*,8*S*,8a*R*)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2*R*,4*R*)-tetrahydro-4-hydroxy-6-oxo-2*H*-pyran-2-yl]ethyl]-1-naphthyl 2-methylbutanoate. Silylation agents, organoboronic acids and trimethylsilyldiazomethane (TMS-DAM) were purchased from Tokyo Kasei Kogyo Co. Ltd. All other chemicals were of reagent grade from commercial sources. Bond Elut C8 (no. 606203), SI (no. 601303) and NH2 (no. 611101) columns were purchased from Analytichem International.

Analytical conditions

The GC/MS system was a Jeol JMS-DX303HF coupled to an HP 5890A gas chromatograph equipped with an HP 7673A automatic sampler. A DB-1 fused-silica capillary column (10 m, 0.25 mm i.d., 0.25 μm film thickness) from J & W Scientific was used. The injection port temperature was 400 °C. The column oven temperature was initially maintained at 100 °C for 1 min and was programmed with an increase of 20 °C min^{-1} to 280 °C and of 10 °C min^{-1} to 320 °C. The temperature was kept at 320 °C for 3.5 min. The ion source

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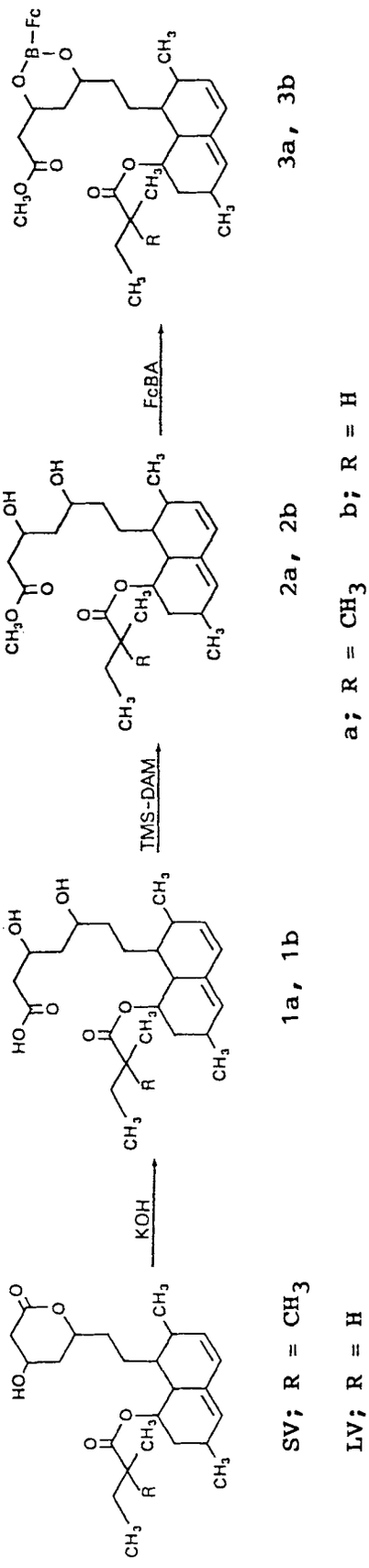

Figure 1. A route for preparing the ferroceneboronates from SV and LV for use as the internal standard, where TMS-DAM indicates trimethylsilyldiazomethane, and FcBA, ferroceneboronic acid.

temperature was 275 °C, the emission current 300 μA, and the electron energy 70 eV. A conversion dynode detector was operated at an accelerating voltage of 10 kV. Helium was used as a carrier gas at a flow rate of 1 ml min^{-1}. All samples (1 μl) were injected with the autosampler, employing a splitless injection method. The split valve was stopped before injection and opened 1 min after injection. All mass spectral data were acquired and processed on a Jeol JMA-DA5500 data system.

Clean-up procedures and measurements

Figure 1 shows the route for derivatizing SV and acid **1a**. Plasma samples, stored at −80 °C until assay, were worked up by the method described below. LV (10 ng) was used as an internal standard (IS) for estimation of SV, and compound **1b** (10 ng of the ammonium salt) for acid **1a**. In these procedures, solvents were removed below 40 °C under reduced pressure, unless otherwise stated.

To a mixture of a plasma sample (1 ml) and saline (1 ml), LV and compound **1b** were added. The contents were applied to a Bond Elut C8 column. After washing with saline (1 ml), water (3 ml) and methanol–water (3 ml, 3:7, v/v), the column was eluted with methanol–water (1 ml, 7:3, v/v) and then with acetonitrile (1 ml). The former eluate contained acids **1a** and **1b**, and the latter, lactones SV and LV.

The former fraction was evaporated to dryness. The residue, dissolved in methanol–tetrachloroethylene (0.1 ml, 1:2, v/v), was treated with TMS-DAM (0.02 ml, 10% in hexane) for 30 min at room temperature. The mixture was passed through a Bond Elut SI column with ethyl acetate–hexane (1 ml, 1:4, v/v). The column was washed with the same solvent (3 ml), and eluted with ethyl acetate–hexane (3 ml, 1:2, v/v). The eluate was concentrated to dryness. A ferroceneboronic acid (FcBA) solution (0.05 ml, 5 mg ml^{-1} in dry pyridine) was added to the residue. The mixture, placed in a screw-capped vial, was warmed at 50 °C for 30 min. Then it was cooled to room temperature, after which the solution was added to a Bond Elut NH2 column. Derivatives **3a** and **3b** were eluted with ethyl acetate–pyridine (1.6 ml, 99:1, v/v). The eluate was concentrated under nitrogen. The residue was reconstructed in an *N*-methyl-*N*-trimethylsilylacetamide (MTMSA)–toluene mixture (0.05 ml, 1:9, v/v) for an assay sample.

The latter lactone fraction was treated with a potassium hydroxide solution (0.5 ml, 0.05 N) for 15 min at room temperature. After acidification with acetic acid (1 ml, 0.1 M), the mixture was applied to a Bond Elut C8 column. The column was washed with water (3 ml) and methanol–water (3 ml, 3:7, v/v) and then eluted with acetonitrile–water–pyridine (1.2 ml, 90:10:1, v/v/v). The solvent was distilled off, and the residue was treated by the method described for the former fraction.

Plasma samples taken from volunteers dosed with 10 mg of SV were assayed. The results are shown in Table 1.

Calibration curves

Procedures for preparing calibration curves were virtually identical to the method described above, except for the treatment of blank plasma (1 ml) containing SV and acid **1a** (as the ammonium salt) at a known concentration, given in Table 2.

The calibration curves for the drugs are linear for their concentrations from 0.1 to 10 ng ml^{-1}. The equation of a calibration curve for SV is $Y_s = 0.752X_s + 0.0185$ ($r = 0.9993$) and that for acid **1a**, $Y_a = 0.704X_a + 0.0106$ ($r = 0.9996$), where Y_s (Y_a) represents the ratio of the peak area of SV (acid **1a**) to that of LV (compound **1b**), and X_s (X_a) stands for the concentration of SV (acid **1a**) in nanograms per millilitre. The equations were derived from the results of the analysis carried out in the same run for determination of the precision of this assay method (Table 2). A slight difference between the slopes of the equations was observed, but this discrepancy is not significant because the SIM assay of plasma spiked with either the lactones (SV, LV) or the acids (**1a**, **1b**) disproved any interconversion of the lactones and acids occurring as a result of the sample processing.

SIM chromatograms

Comparison of SIM chromatograms from blank plasma and from plasma containing the drugs indicated the

Table 1. Plasma concentrations of SV and acid 1a (ng ml^{-1}) after oral administration of SV (10 mg/man)

Time (h)	Simvastatin Subject no. F6	C3	D4	Acid 1a Subject no. F6	C3	D4
0.5	6.07	1.02	0.66	0.17	ND	ND
1	4.96	0.76	0.69	0.22	0.26	0.07
2	2.69	0.33	0.63	0.35	0.29	0.09
3	0.94	0.36	0.54	0.55	0.21	0.17
4	0.72	0.21	0.52	0.52	0.45	0.28
6	0.30	0.09	0.79	0.44	0.20	0.31
8	0.14	ND	0.31	0.57	0.19	0.38
12	ND	ND	0.38	0.16	ND	0.38
24	ND	ND	0.14	0.15	ND	0.31

ND, not detected.

Table 2. Precision of the assay of SV and acid 1a at various concentrations in human plasma ($n = 6$)

Compound	Amount added (ng ml^{-1})	Mean found ±SD (ng ml^{-1})	CV (%)
Simvastatin	0.1	0.10 ± 0.011	11.0
	0.5	0.50 ± 0.018	3.6
	1	1.04 ± 0.046	4.4
	5	5.02 ± 0.207	4.1
	10	9.74 ± 0.171	1.8
Acid **1a**	0.1	0.10 ± 0.008	8.0
	0.5	0.47 ± 0.029	6.2
	1	0.97 ± 0.071	7.3
	5	4.99 ± 0.60	1.2
	10	10.53 ± 0.343	3.2

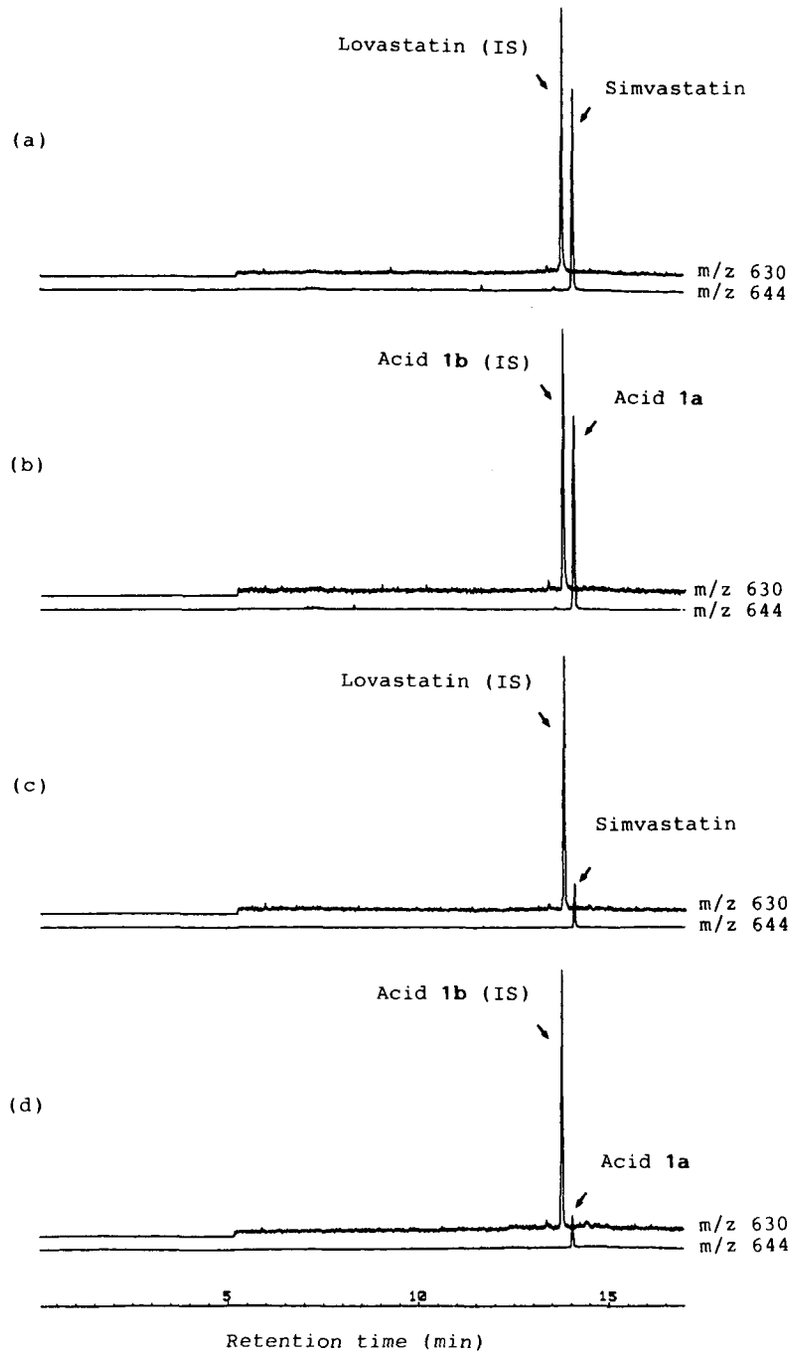

Figure 2. Typical SIM profiles: (a) and (b) are from plasma (1 ml) spiked with 10 ng of both components; (c) and (d) are from human plasma at 4 h after an oral intake of SV (20 mg/man).

absence of materials interfering with the quantification. The quantification limit was 0.1 ng ml^{-1} when the signal-to-noise (S/N) ratio in SIM chromatograms was defined as 4:1.

Figure 2(a) is an SIM chromatogram from the lactone fraction of plasma (1 ml) spiked with SV (10 ng) and acid **1a** (10 ng), and Fig. 2(b) is one from the acid fraction of the same source. Figure 2(c) and (d) are from a lactone and an acid fraction, respectively, of a plasma sample taken from a volunteer 4 h after drug administration (20 mg), showing that the concentration of SV was 2.3 ng ml^{-1} and that of acid **1a** was 1.7 ng ml^{-1}.

Precision and recovery

Table 2 shows the precision of the analysis of both drugs. The samples were analysed within 48 h after completion of the sample preparation. When assay samples were allowed to stand at room temperature, SIM analysis showed little loss of derivatives **3a** and **3b** at 48 h. Slight decreases were observed at 72 h, but the ratio of **3a** to **3b** remained constant during this period. In this stability test, tetradecachloro-*m*-terphenyl (10 ng) was added as a standard at the final step of the clean-up procedures.

Recoveries of SV and acid **1a** were evaluated using their butanoate-1-^{14}C-labelled compounds from MSDRL and found to be 54 ± 5.0% and 75 ± 6.2% (mean ± SD, $n = 6$), respectively.

Preparation of derivative 3a

Derivative **3a** was prepared under conditions described in the clean-up procedures. Methylation of compound **1a** (ammonium salt, 50.2 mg) followed by chromatography on a silica gel column gave an oil (**2a**) which on treatment with FcBA (26 mg) formed derivative **3a**. The crude product was chromatographed over silica gel. Elution with hexane–ethyl acetate (5:1, v/v) gave crystals (m.p. 148.5 – 150 °C, yield 51.8 mg, 71%). High-resolution mass spectra: m/z 644.2983 M^+, calculated for $C_{36}H_{49}BFeO_6$; 644.2971.

Table 3. EI fragment ions ($m/z > 300$) of derivatives from ester 2a and either silylation agents or organoboronic acids

Derivative with	Base peak ion, m/z	Fragment ions, m/z (relative intensity, %)
TMS[a]	198	463 (3), 388 (10), 373 (9), 315 (5)
DMiPS[b]	173	519 (1), 491 (9), 416 (1), 373 (46)[f]
MBA[c]	159	358 (19), 340 (3), 325 (7)
PhBA[d]	159	420 (43), 402 (7), 387 (8), 342 (17)
FcBA[e]	644 M^+	528 (15), 463 (8)

[a] Trimethylsilylimidazole.
[b] Dimethylisopropylsilylimidazole.
[c] Methylboronic acid.
[d] Phenylboronic acid.
[e] Ferroceneboronic acid: ferroceneboronate **3b** from the standards gives fragment ions at m/z 528 and m/z 463.
[f] With 2% of the total ion current.

DISCUSSION

Electron impact (EI) fragment ions of silyl ethers and boronates of compound **2a** are given in Table 3. The mass spectral profiles show that only derivative with FcBA generated a high m/z ion with high intensity. Ferroceneboronate **3a** thus produced the base ion at m/z 644 M^+ with 32.3% of the total ion current and small fragment peaks at m/z 528 $[M - C_5H_{11}CO_2H]^+$ and m/z 463 $[M - C_5H_{11}CO_2H - C_5H_4]^+$. Ferroceneboronate **3b** from IS gave a similar mass spectrum where the base ion at m/z 630 M^+ was observed. Hence, the molecular ions of the derivatives were selected for the SIM channels.

Reaction of acid **1a** with TMS-DAM in a mixture of methanol and tetrachloroethylene gave ester **2a** without any side reaction, although the reaction of **1a** in conventional solvents sometimes gave several by-products shown by HPLC.

Vaporization of the ferroceneboronates (**3a**, **3b**) requires high temperatures because of their high molecular weights. This caused **3a** and **3b** to be retained in the splitless injection system. The quantities of **3a** remaining in the system were not negligible, and the extent of the contamination determined the precision of the analysis. The toluene–MTMSA mixture used as a sample solvent reduced the influence of this contamination, and the precision was improved (Table 2). Without any silylation agent, the coefficient of variation at a concentration of 0.5 ng ml^{-1} was over 10% and the coefficient at 0.1 ng ml^{-1} was about 30% for both drugs.

Advantages of on-column injection for analysing small quantities of high-boiling materials have been reported.[10–13] An on-column injection method was found to be conducive to detection of an absolute amount of 0.2 pg of **3a** at an S/N ratio of 4:1, whereas the injection method adopted in this study allowed for detection of 1 pg at the same S/N ratio. However, attempts to quantify the drugs by the on-column injection method failed; less volatile impurities in practical samples caused GC column deterioration, thus resulting in a sharp decline in the ion peaks of derivatives **3a** and **3b**.

In conclusion, the GC/MS/SIM method described in this paper permits the quantification of SV and acid **1a** in human plasma at a quantification limit of 0.1 ng ml^{-1}. Although much time is required for the clean-up procedures and measurements, the assay of plasma samples suggests that this method can be used for pharmacokinetic studies and therapeutic drug monitoring of SV and acid **1a**.

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