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Gas chromatography–mass spectrometric method for quantitative determination in human urine of dicarboxylic (dioic) acids produced in the body as a consequence of cholesterol biosynthesis inhibition

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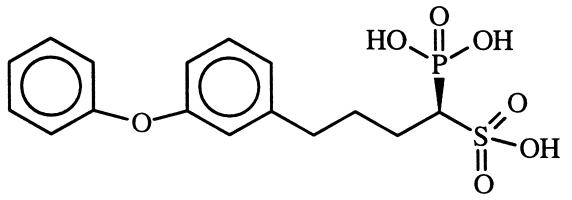
Abstract

A capillary gas chromatography–mass spectrometric (GC–MS) method in human urine has been developed and validated for the quantitative determination of dicarboxylic acids (dioic acids) which are produced in the body as a consequence of the administration of an inhibitor of the enzyme squalene synthase, which is involved in the biosynthesis of cholesterol. The standards and quality control (QC) samples were prepared by adding dioic acids into human urine. Internal standard (sebacic acid) was added to each urine sample (0.1 ml) and then dried by evaporation under nitrogen. The dried sample was reacted with pentafluorobenzyl (PFB) bromide under conditions that maximized the formation of the di-PFB ester (at the expense of the mono-PFB ester) of the dioic acids. After drying by evaporation, each sample residue was reconstituted in mesitylene and injected into a capillary GC–MS system via a splitless injection. The detection was by negative ion chemical ionization mass spectrometry with selected ion monitoring (SIM) of the $[M-PFB]^-$ of the analytes and the internal standard.

Keywords: Dioic acid; Cholestrol

1. Introduction

The blockade of cholesterol biosynthesis has been established as a method for the treatment of hypercholesterolemia in humans [1,2]. BMS-187745 (Fig. 1) blocks cholesterol biosynthesis via the inhibition of the enzyme squalene synthase. This enzyme catalyzes the reductive dimerization of farnesyl diphosphate (FPP) to form squalene, which is subsequently converted to cholesterol [3]. A bioanalytical method, based on HPLC–electrospray mass spectrometry, for the quantitative detelmination



BMS-187745
MW 386.36

Fig. 1. Structure of BMS-187745, an inhibitor of the enzyme squalene synthase.

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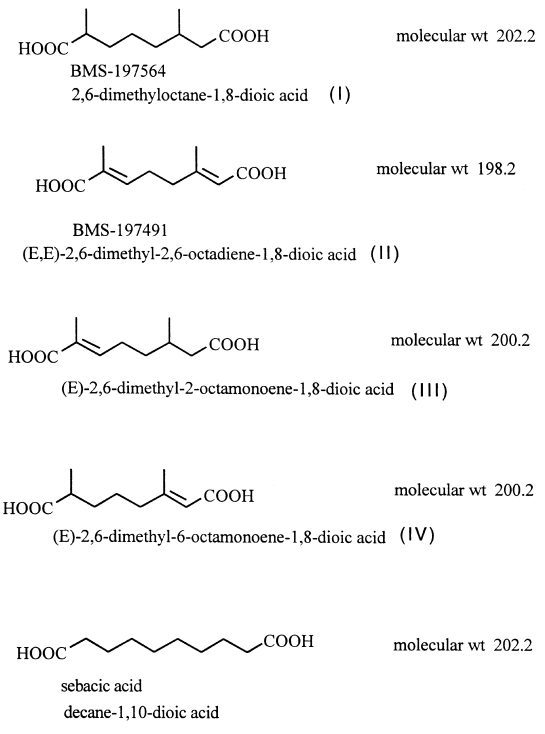


Fig. 2. Structures of the dioic acids BMS-197491, BMS-197564, the two monoene isomers and the internal standard (sebacic acid).

of BMS-187745 in human plasma has been developed and validated [4]. A consequence of blocking the cholesterol biosynthesis pathway via the inhibition of the enzyme squalene synthase is the increased production of farnesol-derived dicarboxylic acids in the body [5–7]. In practice, the presence of any of these 12-, 10- and 7-carbon dicarboxylic acids (dioic acids) in urine at elevated concentrations can be used as a pharmacodynamic indicator of the enzyme inhibition. Thus, during the clinical studies of BMS-187745, urine samples were collected to analyze for dioic acids. In this paper, the GC–MS method developed and validated for the quantification in human urine of two 10-carbon dioic acids (BMS-197491 and BMS-197564, Fig. 2) is presented.

2. Experimental

2.1. Reagents and chemicals

BMS-197491 and BMS-197564 are characterized products of Bristol-Myers Squibb Pharmaceutical Research Institute. Sebacic acid (99%), mesitylene (98%), pentafluorobenzyl bromide (99+%), and *N,N*-diisopropylethylamine (DIPEA) were purchased from Aldrich Chemical (Milwaukee, WI, USA). Methanol, HPLC grade, was obtained from Burdick and Jackson (Muskegon, MI, USA). Acetone, HPLC grade, was from Fisher Scientific (Fair Lawn, NJ, USA). Ammonia (anhydrous), electronic grade (99.999%), and helium, ultrahigh purity grade, were purchased from MG Industries (Linden, NJ, USA). House deionized water, further purified with a Milli-Q water purifying system (Millipore, Bedford, MA, USA), was used. Drug-free human urine was collected from healthy male volunteers in the Department of Metabolism and Pharmacokinetics, Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ, USA). Pentafluorobenzyl bromide (PFBBr) solution for derivatization was prepared fresh daily by dissolving 300 μl of pentafluorobenzyl bromide in 3.7 ml of acetone in an Erlenmeyer flask.

2.2. Equipment

The GC–MS analysis was performed with a Hewlett-Packard (Avondale, PA, USA) 5989A mass spectrometer with a Windows-based (DOS) chemstation. The mass spectrometer was coupled to a Hewlett-Packard 5890 GC, series II, equipped with model 7673A autosampler. The fused silica capillary GC column used was DB-1, 15 m×0.25 mm I.D., 0.25 μm film thickness, from J&W Scientific (Folsom, CA, USA). The splitless inlet liner was 4 mm, silanized, from Restek (Bellefonte, PA, USA). A Turbovap LV evaporator from Zymark (Hopkinton, MA, USA) was used. An incubator, from Boekel Industries Inc., model 132000, was purchased from Baxter Scientific Products (Edison, NJ, USA).

2.3. Chromatographic and mass spectrometric conditions

The injection technique used was the splitless mode. The helium carrier gas flow through the capillary column was 2.0 ml/min (constant flow mode), with a total inlet flow of 50 ml/min. Inlet purge was turned off for 0.70 min after injection. Sample volume injected was 1.0 μl. The column oven temperature was programmed, with initial

temperature of 140°C, initial hold-time of 0.8 min, ramp rate of 35°C/min, final temperature of 260°C, and final hold-time of 1.3 min. Injection temperature of 250°C and interface temperature of 280°C were used. The source and quadrupole temperatures were 200 and 150°C, respectively. The mass spectrometer was used in the negative ion electron-capture chemical ionization mode, with ammonia as the buffer gas. The instrument was tuned daily using fragment ions (m/z 302, 452 and 633) produced by PFTBA. During analysis, a solvent delay of 3.5 min and a dwell time of 25 milliseconds were used. The high mass resolution (0.5 a.m.u.) setting was utilized.

2.4. Standard and QC preparations

Stock solutions of BMS-197491 and BMS-197564 were prepared separately by dissolving accurately weighed reference standard of each compound in methanol (each about 1.0 mg/ml). A portion of each stock solution was transferred to a second volumetric flask and diluted with methanol to obtain a spiking solution that contained both BMS-197491 and BMS-197564 (about 10 μg/ml for each compound). A further diluted spiking solution was similarly prepared (about 1.0 μg/ml for each compound). The calibration set consisted of eight concentrations, each in duplicate, with a range of 100 to 10 000 ng/ml. The standards were prepared from the two spiking solutions. For each standard, a specified volume (ranging from 10 to 100 μl) of the appropriate spiking solution was added to 0.1 ml of blank human urine in a 1-ml autosampler vial. Finally, 50 μl of a solution of sebacic acid (the internal standard) in methanol was added to each vial. The final concentration of the internal standard was 10.1 μg/ml.

Quality control (QC) samples were prepared from BMS-197491 and BMS-197564 methanolic stock and spiking solutions, which were prepared from a second weighing of each reference standard. Four concentrations of QC samples were prepared, at 498, 1990, 5970 and 7960 ng/ml of urine. For each QC sample preparation, a specified volume of the appropriate stock or spiking solution was transferred into a conical-screw cap-tube and the methanol was removed by evaporation. A 10-ml portion of blank urine was then added to obtain a QC sample. All QC samples were stored at −70°C until analysis.

2.5. Derivatization and reconstitution

One hundred-microliter aliquots of each of the four QC levels were pipetted into separate 1-ml autosampler vials. In addition, duplicate QCs (0.1 ml of blank urine with internal standard) and duplicate blanks (0.1 ml of blank urine without internal standard and analyte) were also prepared. Fifty microliters of the internal standard solution in methanol was added to all the samples except the blanks, after which the vials were vortexed. The final concentration of the internal standard was 10.1 μg/ml. The standards, prepared as described above, also contained the same amount of the internal standard. Each standard, QC and blank vial was then evaporated under nitrogen in a Zymark Custom Vial TurboVap at 40°C. This took about 25 min. The samples were then ready for derivatization.

To each dried urine residue, 100 μl of PFBBr solution and 20 μl of DIPEA were added. Each vial was capped, vortexed and incubated for 60 min at 40°C in an incubator. The caps were removed from each vial and the reagents evaporated under nitrogen in a Zymark Custom Vial TurboVap. This took 5 min. The derivatized samples were reconstituted in 500 μl of mesitylene, vortexed for 1 min and capped tightly, ready for injection.

2.6. Analysis

During validation, a set of standards and QC samples were analyzed via single-ion monitoring (SIM) of $[M-PFB]^-$ of the derivatives, at m/z 377.1 for BMS-197491 and m/z 381.1 for BMS-197564 and sebacic acid.

3. Results and discussion

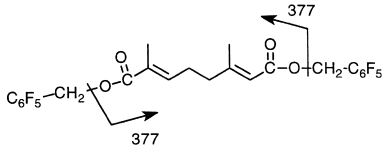
The structures of two dioic acids BMS-197491 and BMS-197564, sebacic acid (internal standard), and two potential dioic acids (*E*)-2,6-dimethyl-2-octamonoene-1,8-dioic acid (monoene isomer III) and (*E*)-2,6-dimethyl-6-octamonoene-1,8-dioic acid (monoene isomer IV) are shown in Fig. 2.

The method was validated for the quantitative determination of BMS-197491 and BMS-197564 in human urine, but the method was not validated for the monoene isomers III and IV since no certified

reference standards were available for the isomers. The only available reference standard for the isomers was a mixture which consisted of four compounds: the two monoene isomers, BMS-197491 and BMS-197564. This mixture was used as a retention time marker and for some qualitative work.

To improve chromatographability and enhance the negative ion chemical ionization response of the dioic acids, the samples were derivatized to form the di-pentafluorobenzyl (di-PFB) esters of the dioic acids. Fig. 3 shows the structure of the di-PFB derivatives and identifies the major fragment ions in the negative ion chemical ionization mass spectrum. The negative ion chemical ionization mass spectra of the di-PFB ester of the dioic acids are shown in Figs. 4–6. The spectra show base peak ions at m/z 377 for BMS-197491, m/z 381 for BMS-19756, and m/z 379 for the monoene isomers. The mass spectrum of the di-PFB ester of sebacic acid (not shown) has the same base peak as BMS-197564 (m/z 381).

There were measurable amounts of endogenous BMS-197491 and BMS-197564 in normal human urine as shown in Figs. 7 and 8. The presence of endogenous dioic acids in blank urine samples used for standard preparation limited the sensitivity of the GC–MS method. However, at the established lower limit of quantitation (LLQ) of 100 ng/ml for BMS-197491 and 110 ng/ml for BMS-197564, the LLQ levels were ≥3 times higher than the endogenous levels. There was little to no response in the internal standard channel of the chromatogram obtained from the blank samples that contained no internal stan-



BMS-197491 di-PFB ester

molecular wt of $CH_2C_6F_5$ (PFB) : 181
molecular wt of BMS-197491: 198
molecular wt of di-PFB ester of BMS-197491: 181+181+198-2 = 558
molecular wt of main fragment of di-PFB ester of BMS-197491: 558 - 181=377

Similarly,

molecular wt of main fragment of di-PFB ester of BMS-197564: 562 - 181=381
molecular wt of main fragment of di-PFB ester of monoenes: 560 - 181=379

Fig. 3. Identification of the major fragment ion of the di-pentafluorobenzyl (di-PFB) ester of BMS-197491, BMS-197564 and the monoene isomers.

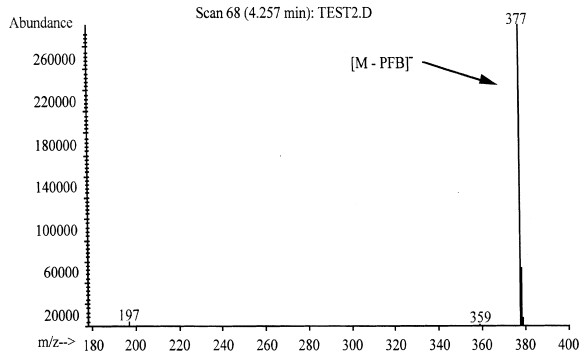


Fig. 4. Negative ion chemical ionization mass spectrum of the di-pentafluorobenzyl (di-PFB) ester of BMS-197491.

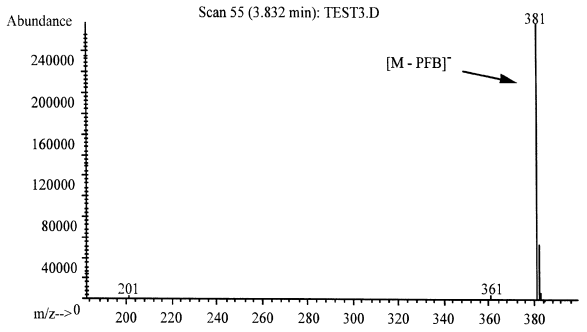


Fig. 5. Negative ion chemical ionization mass spectrum of the di-pentafluorobenzyl (di-PFB) ester of BMS-197564.

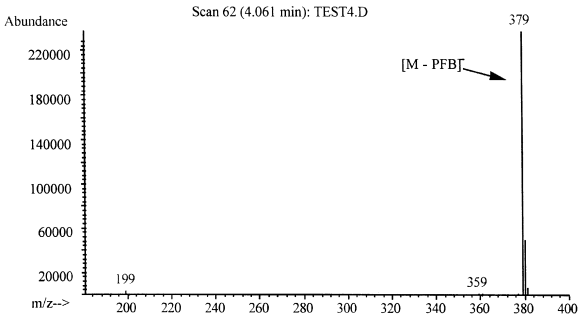


Fig. 6. Negative ion chemical ionization mass spectrum of the di-pentafluorobenzyl (di-PFB) ester of the monoene isomers.

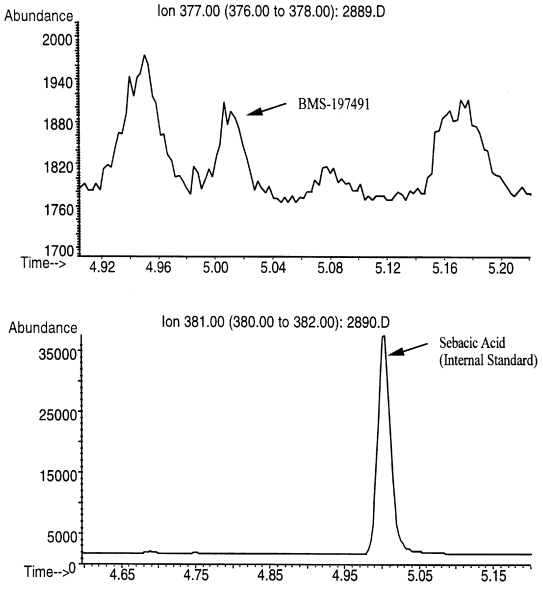


Fig. 7. Chromatogram of BMS-197491 at 0 ng/ml and the internal standard sebacic acid at 10 100 ng/ml of human urine.

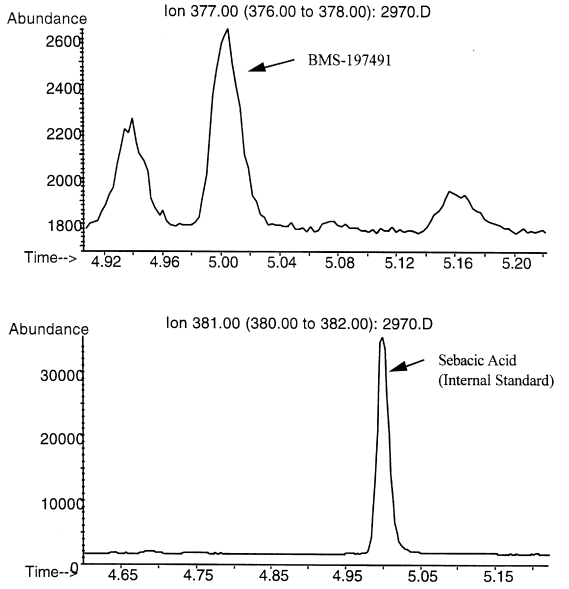


Fig. 9. Chromatogram of BMS-197491 at 100 ng/ml and the internal standard sebacic acid at 10 100 ng/ml of human urine.

dard. Figs. 9 and 10 show the chromatograms of an LLQ sample containing BMS-197491 at 100 ng/ml and BMS-197564 at 110 ng/ml, respectively. The results of the analysis of six different batches of blank human urine samples, each spiked with BMS-197491 and BMS-197564 at LLQ concentrations, are

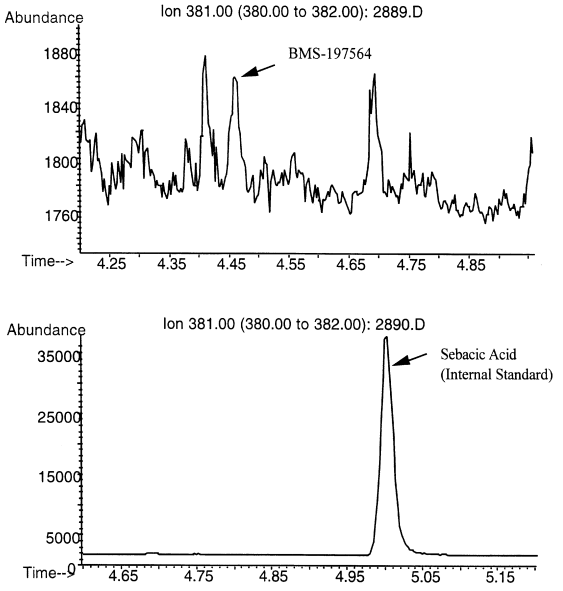


Fig. 8. Chromatogram of BMS-197564 at 0 ng/ml and the internal standard sebacic acid at 10 100 ng/ml of human urine.

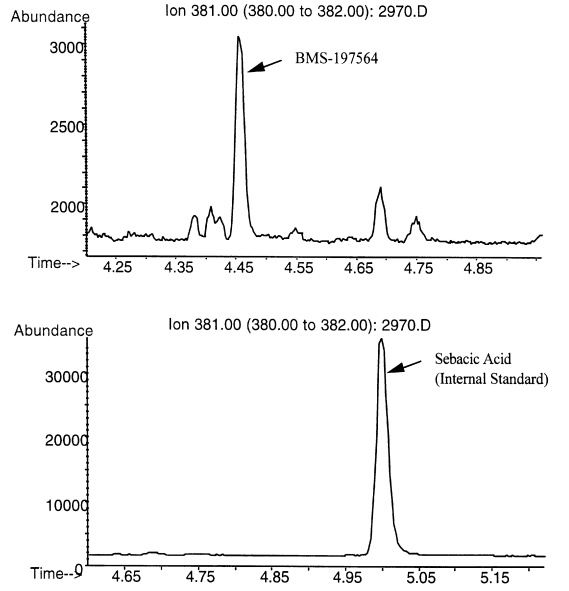


Fig. 10. Chromatogram of BMS-197564 at 110 ng/ml and the internal standard sebacic acid at 10 100 ng/ml of human urine.

Table 1
Lower limit of quantitation (LLQ) for BMS-197491 in human urine

Human urine LLQ sample No.	Deviation (%) from nominal concentration of 100 ng/ml
1	6.4
2	9.9
3	15
4	−9.6
5	6.8
6	18
Mean	108
S.D.	9.7
C.V. (%)	9.0

Table 2
Lower limit of quantitation (LLQ) for BMS-197564 in human urine

Human urine LLQ sample No.	Deviation (%) from nominal concentration of 110 ng/ml
1	10
2	5.5
3	−12
4	−18
5	−21
6	−9.4
Mean	101
S.D.	14
C.V. (%)	14

shown in Tables 1 and 2, respectively. For BMS-197491, the deviation from the nominal concentrations was $\leq \pm 18\%$ and the C.V. was 8.9%. For BMS-197564, the corresponding results were $\leq \pm 21$ and 14%.

Tables 3 and 4 show typical standard curves generated in human urine during validation, fitted to $1/x$ weighted linear regression. The deviation of the back-calculated concentrations from the nominal concentrations was $\leq \pm 15\%$ for BMS-197491 and $\leq \pm 18\%$ for BMS-197564. The C.V. of the duplicate points at each level was $\leq \pm 14\%$ for both analytes.

Accuracy and precision for the determination of BMS-197491 and BMS-197564 in human urine were assessed at four levels of the analytes on three separate days. The results are given in Tables 5 and 6. The precision for both analytes was good as the between-day and within-day C.V. was $\leq 10\%$ at all levels. The deviation of the mean predicted concentrations from the nominal concentration was also $\leq 10\%$ at all levels.

BMS-191491 and BMS-197564 in human urine were found to be stable at −20°C for at least 4 months at all concentrations evaluated. BMS-197491 and BMS-197564 in human urine were stable at room temperature and 4°C for at least 6 h. BMS-197491 and BMS-197564 in human urine were stable when they were subjected to a five-cycle freeze–thaw process. Reconstituted derivatized urine samples that contain both BMS-197491 and BMS-197564 were stable at room temperature for at least 24 h.

Table 3
Linear regression analysis results of a typical standard curve of BMS-197491 in human urine

Nominal concentration (ng/ml)	Deviation from nominal concentration (%)	C.V. of the duplicate points (%)
100	8.1, 12	2.7
200	−9.2, −15	4.8
500	−3.1, 0.6	2.7
1000	−4.0, 2.4	4.6
2000	5.1, 7.2	1.4
4000	5.9, 6.3	0.23
6000	−13, −12	0.79
10 000	−0.1, 7.8	5.5

The two values given in column 2 are for the duplicate standards at each level. The regression parameters are: intercept: 0.00962; slope: 0.0000746; R^2: 0.997.

Table 4
Linear regression analysis results of a typical standard curve of BMS-197564 in human urine

Nominal concentration (ng/ml)	Deviation from nominal concentration (%)	C.V. of the duplicate points (%)
110	14, 9.7	2.4
219	−1.0, −14	9.6
546	−7.0, −0.9	4.5
1100	2.9, 7.8	3.3
2190	−8.7, 1.5	7.6
4380	−18, −0.5	14
6570	6.0, 9.1	2.0
11 000	−0.4, −0.4	0.0

The two values given in column 2 are for the duplicate standards at each level. The regression parameters are: intercept: −0.00404, slope: 0.000234, R^2: 0.994.

Table 5
Summary of the results of BMS-197491 human urine QC samples analyzed on three different days

Nominal concentration (ng/ml)	Deviation (%)	Inter-day precision C.V. (%)	Intra-day precision C.V. (%)
498	10	4.3	4.1
1990	4.6	8.9	6.4
5970	2.9	10	4.1
7960	0.5	1.4	6.0

Although it was not validated for the quantitative determination of the monoene isomers III and IV, the method could be used to estimate the concentrations of these compounds. Fig. 11 depicts the partial chromatographic separation of the two compounds under the conditions utilized. The unit area response (area counts/ng) of BMS-197564, with no C=C double bond, was about four times that of BMS-197491, with two C=C double bonds. In the absence of a suitable reference standard for the monoene isomers, this structure–response relationship was used as the basis for utilizing the BMS-197491 standard curve to estimate the concentration of the monoene isomers in some unknown samples. The area response of each monoene isomer, each with a single C=C double bond, was taken to be two times that of the BMS-197491.

Esterification of carboxylic acids with PFBBr was employed to allow sensitive detection by negative ion electron-capture GC–MS [8]. In the early stage of the method development, the reaction of the dioic acids with PFBBr was studied. Under the conditions chosen for derivatization (60 min, 40°C), the formation of the di-PFB ester appeared to be almost

Table 6
Summary of the results of BMS-197564 human urine QC samples analyzed on three different days

Nominal concentration (ng/ml)	Deviation (%)	Inter-day precision C.V. (%)	Intra-day precision C.V. (%)
498	8.0	5.3	4.7
1990	8.7	0.0[a]	7.0
5970	6.7	0.0[a]	9.4
7960	−5.7	8.6	4.9

[a] No significant additional variation was observed as a result of performing the assay on different days.

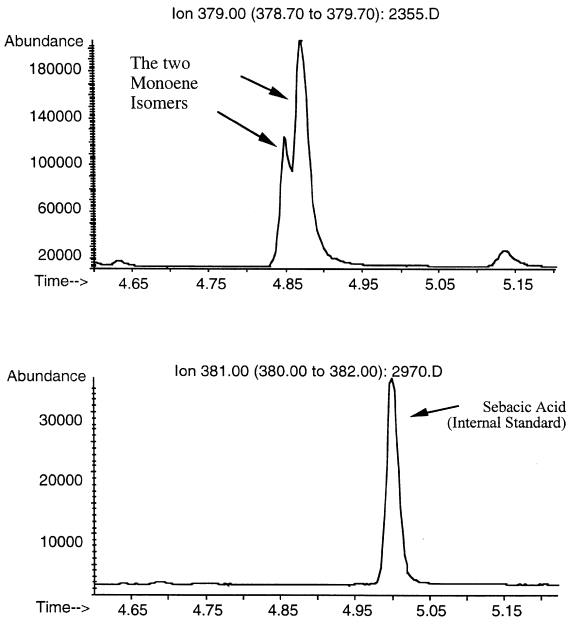


Fig. 11. Chromatogram demonstrating the partial chromatographic separation of the two monoene isomers in human urine.

complete as evidenced by the flattening of the reaction time–di-PFB ester yield curve (Fig. 12). Under milder conditions, significant amounts of the mono-PFB esters, which eluted earlier than the di-PFB ester, were formed. Little or no mono-PFB ester was formed with the procedure described in the method.

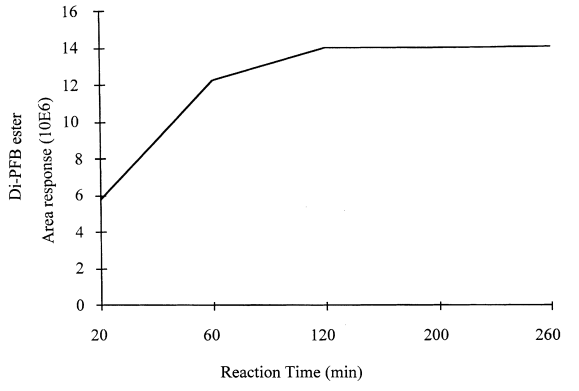


Fig. 12. Reaction of the dioic acids with pentafluorobenzyl bromide.

The chromatographic retention time (5.0 min) of sebacic acid (a straight-chain 10-carbon dicarboxylic acid, with no C=C double bond) was longer than the retention time (4.5 min) of BMS-197564 (a branched-chain 10-carbon dicarboxylic acid, with no C=C double bond). This longer retention time for the straight-chain isomer than for the branched-chain isomer was expected. The retention time (5.0 min) of BMS-197491 (a branched chain 10-carbon dicarboxylic acid, with two C=C double bonds) was longer than the retention time (4.5 min) of BMS-197564 (a branched chain 10-carbon dicarboxylic acid, with no C=C double bond). This was not expected on the nonpolar DB-1 column employed.

4. Conclusion

We have developed and validated a rugged capillary GC–MS method for the quantitative determination in human urine of two dioic acids, BMS-197491 and BMS-197564. The method developed could also be used to estimate the concentrations of other dioic acids for which reference standards were not readily available. The sensitivity of the method was limited by the endogenous concentrations of the dioic acids in human urine. Accordingly, the method was validated with a lower limit of quantitation of 100 ng/ml. The urine sample was simply dried by evaporation prior to the derivatization reaction to form the di-PFB esters of the analytes and the internal standard. Under the conditions utilized, there was little or no formation of the mono-PFB esters. The method was used for analysis of human urine samples from two large clinical studies. The results of the dioic acid concentrations and their pharmacokinetic and mechanistic implications are to be presented elsewhere.

Acknowledgements

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