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Quantitative analysis of HBY 097 and its metabolites in human serum and urine by HPLC[1]

V.K. Agarwal [a,*], G.J. Krol [a], V. Krone [b], D. Roberts [a]

[a] *Clinical Pharmacology, Bayer Corporation, 400 Morgan Lane, West Haven, CT 06516-4174, USA*
[b] *HMR Preclinical Development, Hoechst AG, D-65926 Frankfurt, Germany*

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Abstract

Two HPLC methods were developed: one for the quantitation of HBY 097 reverse transcriptase inhibitor and its metabolites M2 and M3 in human serum, and one for the quantitation of metabolite M5 in urine. The HPLC procedure for the quantitation of HBY 097 and its metabolites M2 and M3 in human serum involved protein precipitation with acetonitrile followed by automated on-line trace enrichment. The HPLC procedure for the analysis of metabolite M5 in urine involved enzymatic hydrolysis of urine with β-glucuronidase to convert metabolite M5 (glucuronide of M3) to M3. Reverse phase chromatographic separation with gradient elution, UV detection at 335 nm, and internal standard were used to quantitate analytes in both procedures. The lower quantitation limits were 25 ng ml^{-1} for HBY 097 and metabolites M2 and M3 in serum, and 0.5 μg ml^{-1} for the metabolite M5 in urine measured as metabolite M3 after hydrolysis. The HBY 097 and metabolite M3 concentrations were specific but metabolite M2 was semi-specific because the two diastereomers of M2 were not resolved by the present chromatographic procedure. Both procedures were applied to the quantitation of HBY 097 and its metabolites in serum and urine of HIV positive patients who were enrolled in a clinical study of drug safety and pharmacokinetics. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: HBY 097; Reverse transcriptase inhibitor; On-line sample enrichment, HPLC; Metabolites of HBY 097; Analysis of serum and urine

1. Introduction

HBY 097, a non-nucleoside reverse transcriptase inhibitor, is the *S*-enantiomer of a quinoxaline derivative. This compound is specifically active against HIV-1 reverse transcriptase (RT) by an allosteric mechanism in nanomolar concentration. Appearance of resistant strains is delayed for a longer period, compared with other RT-inhibitors [1].

HBY 097 is presently under Phase I clinical trials, and appears to be safe and reasonably well tolerated up to 1000 mg tid for 14 days [2]. During initial in-vivo studies in rat, dog and

* Corresponding author.

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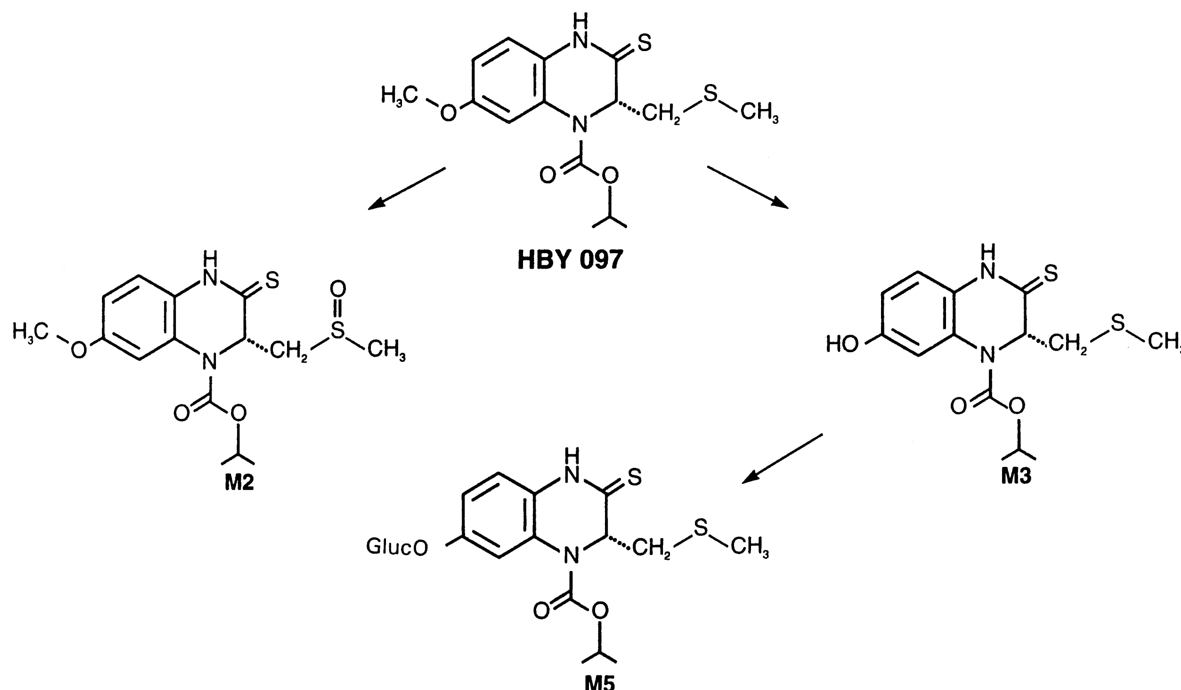

Fig. 1. Structures of metabolites.

monkey, a number of metabolites were observed in urine and plasma. In humans, this compound goes through significant biotransformation and various metabolites have been identified. The structures of the important metabolites are given in Fig. 1.

A HPLC method has been developed for the simultaneous quantitation of HBY 097 and its major metabolites, M2 and M3, in serum. The HBY 097 and metabolite M3 concentrations were specific but metabolite M2 was semispecific because two diastereomers of M2 were not resolved by this chromatographic procedure. Another HPLC method for the quantitation of the major metabolite M5 in urine—a glucuronide conjugate of M3 was also developed. Since no reference standard of metabolite M5 was available, quantitation of metabolite M5 was performed after enzymatic hydrolysis to metabolite M3.

The pharmacokinetic data obtained using these procedures has already been presented at the XI International Conference on AIDS [2].

2. Experimental

2.1. Materials and reagents

Analytical grade potassium dihydrogen phosphate, potassium hydroxide, anhydrous ethanol, and HPLC grade acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ). β-glucoronidase (780 000 units g^{-1}) was purchased from Sigma (St. Louis, MO). A Milli-Q reagent water system (Millipore, Bedford, MA) was used for the purification of deionized water. Hypersil C-18, 5 μm, 250 × 4.6 mm analytical columns were purchased from Phenomenex (Torrance, CA). The HPLC system consisted of a Gilson 233 XL autosampler with on line column switching for trace enrichment, Perkin Elmer LC 200 binary solvent delivery system and LC95 UV detector. Trace enrichment cartridges (TEC), PreluteTM 3, 5.8 × 4.6 mm, 10 μm, 70 mg were purchased from Gilson Medical Electronics (Middleton, WI). Human serum was purchased from Biological Speciality (Colmar, PA) and urine was collected from

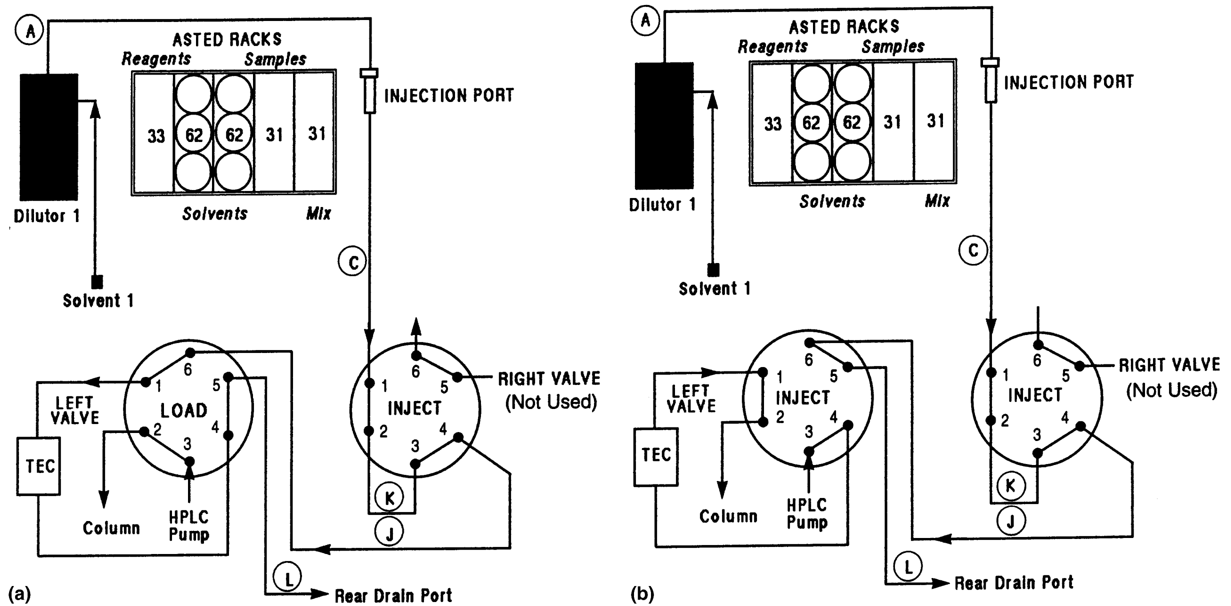

Fig. 2. (a) Schematic of the on-line trace enrichment system in sample enrichment mode; (b) schematic of the on-line trace enrichment system in backflush mode.

Table 1
HPLC gradient for the analysis of HBY 097 and metabolites M2 and M3 in serum

Time (min)	Flow rate (ml min^{-1})	Solvent (10 mM KH_2PO_4) (%)	Solvent B (acetonitrile) (%)	Gradient
8.5	1.1	65	35	
10.0	1.25	48	52	Linear
9.5	1.25	48	52	
1	1.4	65	35	Linear
3.5	1.4	65	35	
1	1.1	65	35	

in-house volunteers. Reference compounds of HBY 097, metabolites M2, M3, and internal standard were obtained from Hoechst, Frankfurt, Germany.

Four potassium phosphate buffers, 10 mM (pH 6.0), 10 mM (pH 4.6), 75 mM (pH 6.8), and 200 mM (pH 6.0) were prepared using potassium dihydrogen phosphate and potassium hydroxide. A solution of β-glucuronidase (0.5 mg ml^{-1}) was prepared in the pH 6.8, 75 mM buffer. Reference standard stock solutions of HBY 097 and metabolites M2 and M3 at various concentrations were prepared in ethanol. All stock solutions were stored at 4°C.

2.2. Preparation of serum calibration standards and quality control samples

Calibration standards and quality control samples were prepared by spiking blank serum and urine with the appropriate amount of stock solutions. Different stock solutions were used to prepare calibration standards and quality control samples. Serum calibration levels were 25, 50, 100, 250, 1000, 2000 and 5000 ng ml^{-1}, and serum quality control levels were 40, 75, 500 and 3000 ng ml^{-1} for all three analytes (HBY 097, metabolite M2 and M3). Urine calibration levels were 0.5, 2, 5, 25, 75 and 200 μg ml^{-1} and urine quality

Table 2
HPLC gradient for the analysis of metabolite M5 in urine

Time (min)	Flow rate (ml min^{-1})	Solvent A (10 mM KH_2PO_4) (%)	Solvent B (acetonitrile) (%)	Gradient
2.0	1.1	80	20	
10	1.1	70	30	Linear
5	1.1	70	30	
10	1.25	48	52	Linear
9.5	1.25	48	52	
1	1.4	80	20	Linear
3.5	1.4	80	20	
0.5	1.1	0	0	

control levels were 1, 50 and 160 μg ml^{-1} of metabolite M3. Spiked serum (500 μl) and 250 μl of spiked urine were aliquoted into 13 × 100 mm screw top tubes. The tubes were capped and stored at −70°C until used.

2.3. Preparation of serum samples

Serum (500 μl) was transferred to a 13 × 100 screw top tube, and 1000 μl of acetonitrile containing internal standard (500 ng ml^{-1}) was added to the tube to precipitate the proteins. The sample tube was vortexed for about 15 s and left at room temperature for 10 min. The sample tube was vortexed again for 15 s and centrifuged at 2000 × g for 10 min at room temperature. The clear supernatant (1.0 ml) was transferred to a 15 ml polypropylene tube and diluted with 1.6 ml of 10 mM, pH 6.0 buffer. After vortexing, the sample was transferred to an autosampler vial and loaded onto the autosampler cooled to 4°C.

2.4. Preparation of urine samples

Urine (250 μl) was transferred to a 13 × 100 screw top tube, and 250 μl of β-glucuronidase solution prepared in 75 mM, pH 6.8 buffer was added to the tube. The sample tube was capped, vortexed, and incubated at 37°C for 30 min in a water bath. A 100 μl portion of internal standard spiking solution was added to the tube and vortexed. The sample was diluted with 400 μl of 200 mM, pH 6.0 potassium phosphate buffer and transferred to sealed autosampler microvials. A 100 μl portion was injected on to HPLC.

2.5. Automated trace enrichment of serum samples

The trace enrichment cartridge was first conditioned with 4 ml of 10% aqueous acetonitrile using an automated Gilson on-line column switching system at a flow rate of 0.5 ml min^{-1}, followed by 2 ml of 10 mM, pH 6.0 potassium phosphate buffer at a flow rate of 0.5 ml min− 1. Fig. 2(a) shows the schematic of the on-line trace enrichment system in the sample enrichment mode. A 300 μl portion of the sample prepared after protein precipitation was loaded onto the cartridge. Analytes HBY 097, metabolites M2 and M3, and internal standard were retained on the cartridge during the enrichment and were backflushed with the initial chromatographic solvent (Table 1) onto the analytical column. Fig. 2(b) shows the schematic of the on-line trace enrichment system in the backflush mode.

2.6. Chromatography

The Hypersil, C-18, 5 μm, 250 × 4.6 mm analytical column was used for chromatography at ambient temperature. A gradient elution was necessary to separate all the analytes from the endogenous constituents of the serum and urine. Tables 1 and 2 describe the gradient conditions used for the analysis of serum and urine samples, respectively. Detection was performed using UV at 335 nm.

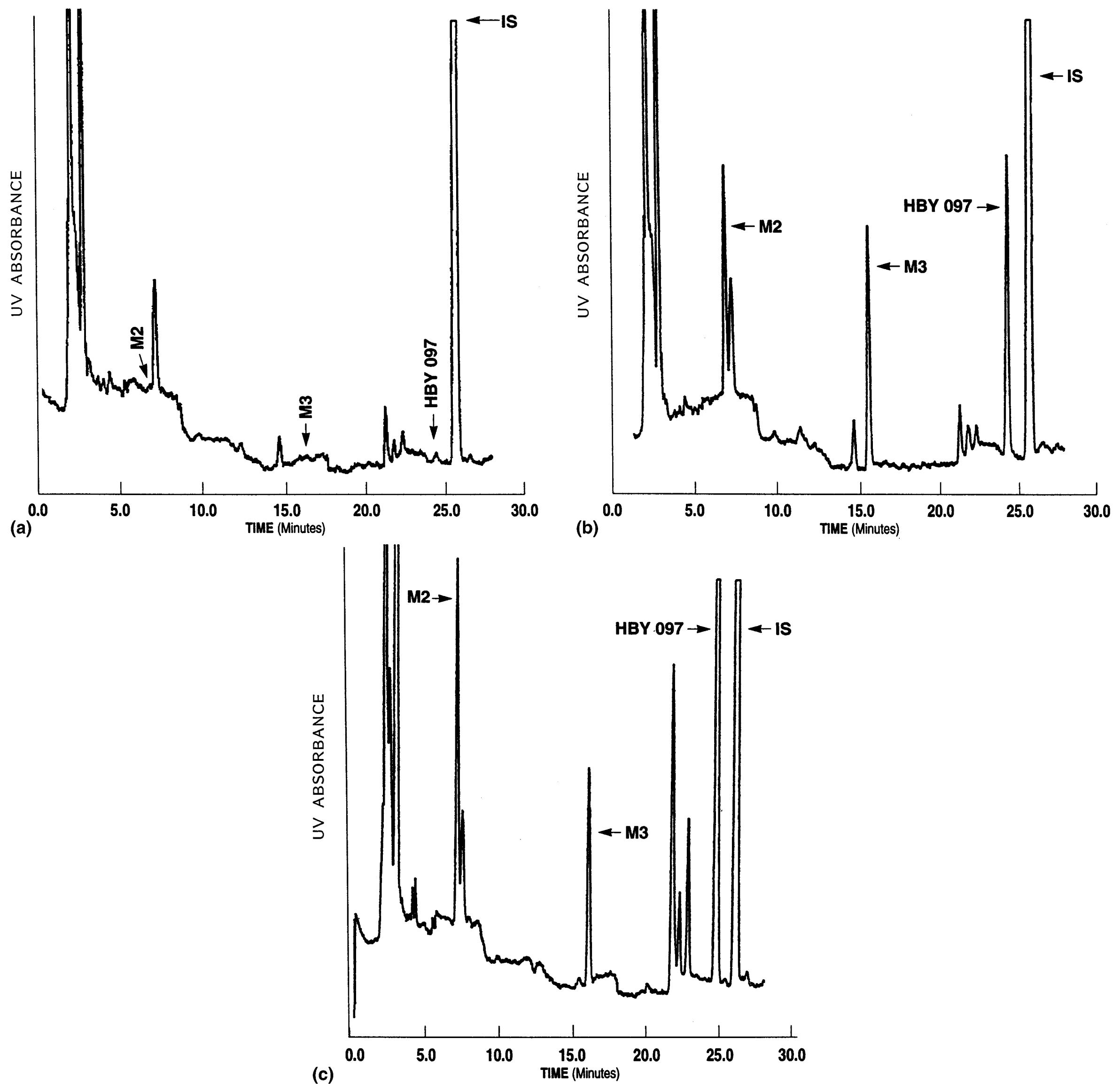

Fig. 3. (a) HPLC chromatogram of blank serum with internal standard; (b) HPLC chromatogram of calibration standard containing 100 ng ml^{-1} of HBY 097 and metabolites M2 and M3; and (c) HPLC chromatogram of post-dose subject serum sample containing 505 ng ml^{-1} of HBY 097, 172 ng ml^{-1} of metabolite M2 and 95 ng ml^{-1} of metabolite M3

3. Results and discussion

3.1. Chromatographic specificity

Fig. 3(a–c) illustrate the representative chromatograms of serum calibration standards and subject serum sample. No interferences were observed at the retention times of metabolite M3 and internal standard. Negligible interference was observed at the retention time of HBY 097 and did not affect quantitation. An endogenous peak was observed close to the retention time of

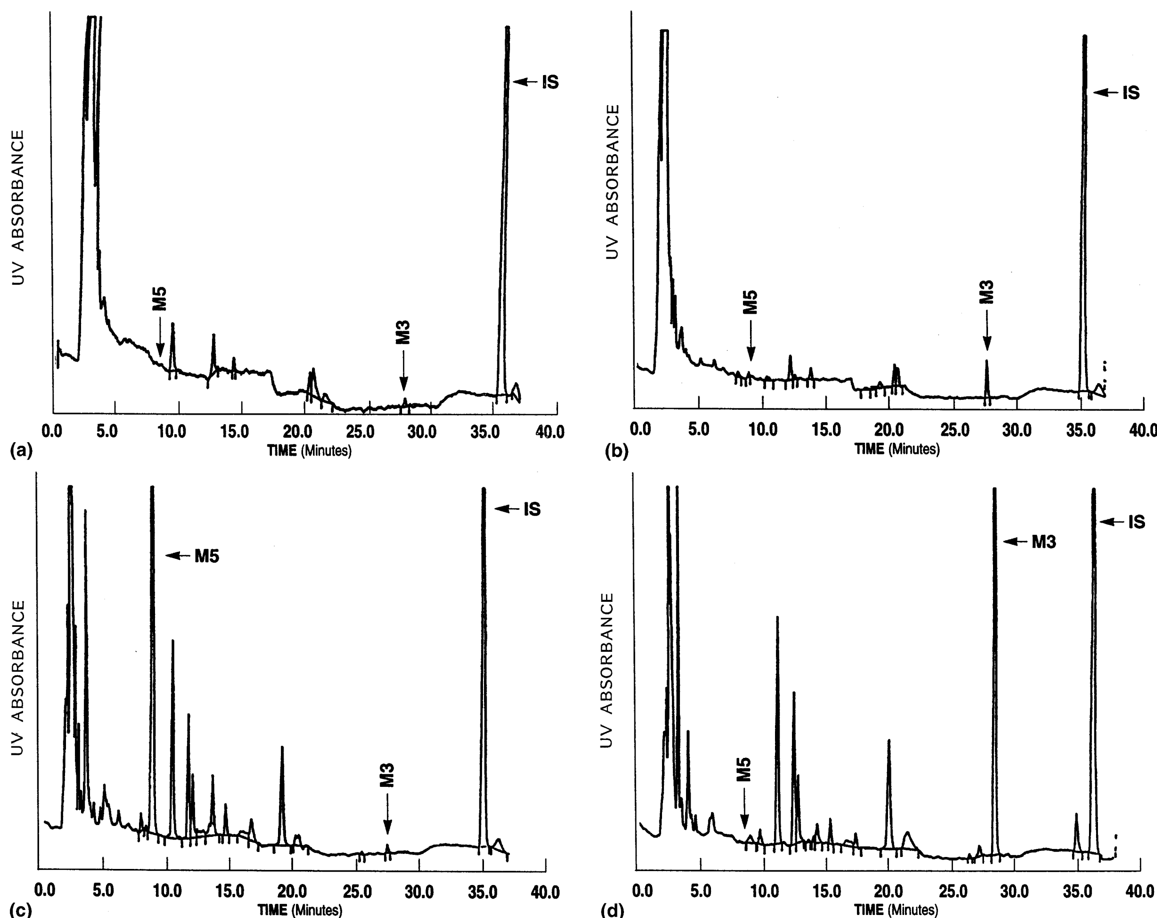

Fig. 4. (a) HPLC chromatogram of blank urine with internal standard; (b) HPLC chromatogram of urine calibration standard containing 0.5 μg ml^{-1} of metabolite M3; (c) HPLC chromatogram of post-dose subject urine sample before hydrolysis; and (d) HPLC chromatogram of post-dose subject urine sample after hydrolysis

metabolite M2 but the incomplete resolution of metabolite M2 from this endogenous peak did not affect quantitation. Two diastereomers of metabolite M2 were not resolved by this chromatographic system and consequently, only the sum of the two diastereomers was determined. A chromatographic system which resolves two diastereomers of metabolite M2 was recently developed by Krone [3]. The retention times of HBY 097, metabolites M2, M3, and internal standard were approximately 25, 7, 16 and 26 min respectively. Fig. 4(a–d) illustrate the representative chromatograms of urine calibration standards and subject urine sample. Negligible interferences were observed at the retention times of metabolites M5, M3, and internal standard in blank urine sample (Fig. 4(a)). Subject urine sample (Fig. 4(c)) also showed a small peak (less than 0.1 μg ml^{-1}) at the retention time of metabolite M3. This peak was a combination of the interference observed in blank urine sample and trace of native metabolite M3. The presence of this peak however did not

Table 3
Intra-day precision and accuracy observed with serum calibration standards containing HBY 097 and metabolites M2 and M3

Analyte	Theoretical conc. (ng ml^{-1})	Mean observed[a] conc. (ng ml^{-1})	RSD (%)	Accuracy (%)
HBY 097	25.0	24.9	5.3	99.5
	50.0	50.9	3.2	101.7
	100.0	98.9	1.0	98.9
	250.0	249.8	0.7	99.9
	1000.0	1001	1.1	100.0
	2000.0	1988	1.0	99.4
	5000.0	5022	0.6	100.4
Metabolite M2	25.0	24.9	5.0	99.6
	50.0	50.4	5.2	100.8
	100.0	98.5	3.6	98.5
	250.0	254.4	3.2	101.8
	1000.0	1007	4.8	100.7
	2000.0	2037	3.1	101.9
	5000.0	4835	3.6	96.7
Metabolite M3	25.0	25.0	6.0	99.8
	50.0	49.8	4.8	99.6
	100.0	101.4	2.4	101.4
	250.0	249.6	1.7	99.8
	1000.0	1000	3.0	99.9
	2000.0	2013	1.9	100.6
	5000.0	4939	2.7	98.8

[a] Six replicates at each level.

affect the lower quantitation of 0.5 μg ml^{-1}. The retention times of metabolites M5, M3 and internal standard were approximately 9, 28 and 36 min. The peak for metabolite M5, which was present in the subject's urine sample before hydrolysis (Fig. 4(c)), disappeared completely after hydrolysis (Fig. 4(d)) and showed a concurrent increase in metabolite M3. This observation confirmed the complete conversion by enzymatic hydrolysis of metabolite M5 to M3. Under these chromatographic conditions, it was also possible to quantitate HBY 097 and metabolite M2 in urine. However, the concentration of HBY 097 was negligible and metabolite M2 was not stable during sample collection.

3.2. Accuracy and precision

Tables 3 and 4 summarize the intra-day and inter-day accuracy and precision of the serum assay and Tables 5 and 6 summarize the intra-day and inter-day accuracy and precision for urine assay. The intra-day accuracy and precision were based on the analysis of six replicates of calibration standards at seven concentration levels. The inter-day accuracy and precision were obtained with quality control samples which were analyzed concurrently with the subject samples. All quality control samples were within 15% of the nominal values.

3.3. Quantitation

The lower and upper quantitation limits of the serum assay were 25 ng ml^{-1} and 5000 ng ml^{-1} for all three analytes. These limits were selected based on the expected serum concentrations in subject samples. It was possible to quantitate all the analytes to 10 ng ml^{-1} by loading a higher volume of sample on the trace enrichment cartridge. The lower and upper quantitation limits of the urine assay were 0.5 μg ml^{-1} and 200 μg ml^{-1} for metabolite M5 quantitated as metabolite M3. The quantitation was based on the relative

Table 4
Inter-day precision and accuracy observed with serum quality control samples assayed concurrently with study samples during 15 months

Analyte	Theoretical conc. (ng ml^{-1})	Replicates (n)	Mean observed conc. (ng ml^{-1})	RSD (%)	Accuracy (%)
HBY 097	40.0	42	40.0	4.9	100.0
	75.0	81	72.2	6.6	96.2
	500.0	81	471.5	3.1	94.3
	3000.0	81	2912	2.9	97.1
Metabolite M2	40.0	42	39.7	6.7	99.2
	75.0	81	74.3	7.1	99.1
	500.0	81	499.0	4.5	99.8
	3000.0	81	2982	3.6	99.4
Metabolite M3	40.0	42	41.4	5.4	103.5
	75.0	59	76.6	6.9	102.1
	500.0	59	506.9	2.9	101.4
	3000	59	2979	2.7	99.3

Table 5
Intra-day precision and accuracy observed with urine calibration standards containing metabolite M3

Theoretical conc. (μg ml^{-1})	Mean observed[a] conc. (μg ml^{-1})	RSD (%)	Accuracy (%)
0.50	0.50	6.5	99.3
2.00	2.05	5.8	102.4
5.00	4.98	3.8	99.5
25.0	24.8	3.3	99.2
75.0	74.7	5.8	99.6
150.0	151.2	4.8	100.8
200.0	198.4	4.6	99.2

[a] Six replicates at each level.

peak height response ratio (PHRR) of analyte to internal standard. Regression curves of PHRR vs. concentration were plotted using CONCAL, software developed by the Institute of Clinical Pharmacology, Bayer, AG.

3.4. Recovery

The percent recoveries ranged from 86 to 96% for HBY 097 and metabolites M2 and M3 and 97% for the internal standard after sample preparation involving protein precipitation. The percent recoveries of metabolite M3 and the internal standard from urine were not determined because sample preparation only involved the dilution of subject samples.

3.5. Stability of HBY 097 and metabolites M2 and M3 in serum

The stability of HBY 097 and metabolites M2 and M3 in serum were determined 7 weeks after storage at −20 and −70°C. All three compounds were stable at −70°C. However, at −20°C, an 8% loss in metabolite M2 was observed. Therefore, all the calibration standards, quality control, and subject samples were stored at −70°C. The stability of HBY 097 and metabolites in serum stored at −70°C after three freeze–thaw cycles was also determined. No loss was observed for HBY 097 and metabolite M3. A loss of 15% was observed for metabolite M2 after the third freeze–thaw cycle. All three analytes and internal standard were

Table 6
Inter-day precision and accuracy of metabolite M3 observed with urine quality control samples assayed concurrently with the study samples during 10 months

Theoretical conc. (μg ml^{-1})	Replicates (n)	Mean observed conc. (μg ml^{-1})	RSD (%)	Accuracy (%)
1.0	74	0.99	0.1	99.8
50.0	74	50.3	1.6	100.6
160.0	74	160.4	5.9	100.2

stable in precipitated serum on the autosampler at 4°C after a 24 h period.

3.6. Stability of metabolites M5 and M3 in urine

Because no reference standard was available for metabolite M5, no direct stability data was collected. Quantitation of subject samples after 3 months of storage at −70°C showed no loss of metabolite M5 or increase in the concentration of metabolite M3, thus indicating the stability of M5. Additional stability data was collected for metabolite M3. Metabolite M3 was stable in urine during 140 days of storage at −70°C and in a reconstitution solvent after 24 h on the autosampler at room temperature. The internal standard and metabolite M3 were found to be unstable in the reconstituted solvent after prolonged (over 24 h) exposure to the air. Exposure of the sample to air was minimized by filling autosampler vials to maximum capacity.

4. Conclusions

The procedures described in this report are specific, accurate, precise, and sufficiently sensitive for the quantitation of HBY 097, metabolite M3 and the sum total of metabolite M2 diastereomers in serum and metabolite M5 in urine samples. The manual sample preparation steps involve only precipitation and/or dilution. Sample enrichment prior to chromatography is automated. These procedures were suitable for the analysis of samples collected during pharmacokinetic studies.

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