DIONEX

Application Note 257

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HPLC Assay Method for Drug Products Containing Anti-Tuberculosis Active Pharmaceutical Ingredients

INTRODUCTION

Isoniazid, pyrazinamide, rifampicin, and ethambutol are anti-tuberculosis compounds. The standard treatment for tuberculosis (TB) is to treat the patient with a combination of these four compounds for two months, followed by isoniazid and rifampicin alone for an additional four months. Depending on the state of infection, ethambutol may be omitted from the treatment.[1] These compounds are used in combination because they have different modes of action. For more than 50 years, TB has been treated with combination drug therapy and there are a number of available combination drug products with different drug contents and composition.

The United States Pharmacopeia (USP) 32 NF27 contains a monograph for isoniazid, pyrazinamide, rifampicin, and ethambutol hydrochloride tablets.[2] The monograph has two assay methods for this drug product. One method is for an assay of isoniazid, pyrazinamide, and rifampicin and the other for ethambutol. Both are HPLC methods.

The work shown here reports a single HPLC assay method that accurately determines these four compounds. The method is evaluated using two drug products. One drug product is a tablet containing all four compounds and the other drug product contains three of the compounds (no ethambutol). The 10 min HPLC method accurately determines all compounds of interest in both drug products. This method saves time, reduces mobile phase consumption, and reduces waste. Further savings and waste reduction are possible with an ultra HPLC (UHPLC) method that requires less than 2 min per injection.

EQUIPMENT

Dionex UltiMate® 3000 system including:

Equipment	Conventional HPLC	UHPLC
Integrated vacuum degasser solvent rack	SRD-3600	SRD-3600
Pump	DGP-3600A	HPG-3400RS
Split-loop sampler	WPS-3000TSL	WPS-3000TRS
Column compartment	TCC-3200	TCC-3000RS
Diode array detector	PDA-3000	DAD-3000RS
Sample loop size	100 µL	100 µL*
Mixer	Standard	200 µL Static mixer kit
Flow cell	13 µL SST	2.5 µL SST
Chromeleon® Chromatography Data System (CDS) software version	6.80 SR7	6.80 SR7

*While the data collected in this AN used a 100 µL loop, a smaller loop (e.g. 20 µL) would be more appropriate.

REAGENTS AND STANDARDS

Deionized water (DI), Type I reagent grade, 18 MΩ-cm resistivity or better

Acetonitrile (CH_3CN), HPLC grade (LAB-SCAN)

Sodium dihydrogen orthophosphate, AR grade (Ajax)

Triethylamine (TEA), AR grade (Fisher)

Orthophosphoric acid, (85%), AR grade (ASP Finechem)

Isoniazid, (101.30%), (provided by customer)

Pyrazinamide, (99.91%), (provided by customer)

Ethambutol, (98.10%), (provided by customer)

Rifampicin, (96.92%), (provided by customer)

CONDITIONS

Conventional HPLC

Column: Acclaim® Polar Advantage II (PA2), 3 µm 4.6 × 150 mm (P/N 063191)
Acclaim PA2 Guard, 5 µm 4.3 × 10 mm (P/N 063195)
Acclaim Guard Kit (P/N 059526)

Mobile Phase: A: 8% CH_3CN in 20 mM NaH_2PO_4 (plus 1.5 mL TEA per liter), pH 6.8
B: 50% CH_3CN in 20 mM NaH_2PO_4 (plus 1.5 mL TEA per liter), pH 6.8

Flow Rate: 1.0 mL/min

Gradient: 100% A from -5 to 3 min, ramp to 100% B in 0.5 min, and hold 100% B for 7 min

Column Temp.: 35 °C

Injection Volume: 5 µL

Detection: Channel_1 UV-vis_1 at 200 nm and 337 nm at 5 min
Channel_2 UV-vis_2 at 238 nm
Wavelength scanning 190 to 800 nm
Data collection rate 5 Hz, rise time 0.5 s

UHPLC

Column: Acclaim PA2, 2.2 µm 2.1 × 100 mm (P/N 068990)
EXP™ Pre-Column Ultra High Pressure Filter Cartridges, 0.2µm (P/N 15-04-03097, Optimize Technologies)
EXP Filter Holder with EXP™ Titanium Hybrid Ferrule (P/N 15-04-03837, Optimize Technologies)
Mobile Phase: A: 4% CH_3CN in 20 mM NaH_2PO_4 (plus 1.5 mL TEA per liter), pH 6.8
B: 50% CH_3CN in 20 mM NaH_2PO_4 (plus 1.5 mL TEA per liter), pH 6.8

Flow Rate: 1.0 mL/min

Gradient: 100% A from -2.5 to 0.5 min, ramp to 100% B in 0.1 min, and hold 100% B for 1.2 min

Column Temp.: 35 °C

Injection Volume: 1.5 µL

Detection: Channel_1 UV-vis_1 at 200 nm and 337 nm at 1 min
Channel_2 UV-vis_2 at 238 nm
Wavelength scanning 190 to 800 nm
Data collection rate 25 Hz, response time 0.2 s

PREPARATION OF SOLUTIONS AND REAGENTS

20 mM NaH_2PO_4 pH 6.8 plus 1.5 mL TEA

Dissolve 3.12 g NaH_2PO_4 in 700 mL water, add 1.5 mL TEA, and mix well. Transfer this solution into a 1 L volumetric flask and add water to bring to volume. Adjust to pH 6.8 with orthophosphoric acid.

Mobile Phases

Mobile Phase A (Conventional HPLC)

Mix 80 mL CH_3CN with 920 mL of 20 mM NaH_2PO_4 TEA pH 6.8. Filter with a 0.2 µM filter.

Mobile Phase A (UHPLC)

Mix 40 mL CH_3CN with 960 mL of of 20 mM NaH_2PO_4 TEA pH 6.8. Filter with a 0.2 µM filter.

Mobile Phase B

Mix 500 mL CH_3CN with 500 mL of 20 mM NaH_2PO_4 TEA pH 6.8. Filter with a 0.2 µM filter.

Stock Standard Solutions

Accurately weigh 20 mg, 110 mg, 74 mg, and 110 mg isoniazid, pyazinamide, ethambutol, and rifampicin, respectively, into a 50 mL beaker. Add 5 mL CH_3CN and 20 mL mobile phase A. Stir and place in an ultrasonic bath until dissolution is complete. Transfer this solution to a 50 mL volumetric flask, rinse the beaker with mobile phase A a few times, and transfer into the same volumetric flask. Add mobile phase A to bring to volume. Table 1 shows the concentration of the stock standard solution.

Working Standard Solutions

Pipet 1, 1.5, and 2 mL stock standard solution into separate 10 mL volumetric flasks. Add mobile phase A to bring to volume. Table 1 shows the concentrations of the working standard solutions.

Note: Prepare stock and working standard solutions just before analysis.

SAMPLE PREPARATION

The authors analyzed two different anti-tuberculosis drug samples (referred to as Samples A and B) and these drugs had different compositions. Sample A contained four active pharmaceutical ingredients (API): isoniazid, pyrazinamide, ethambutol, and rifampicin. Sample B had only three of the APIs; it lacked ethambutol. Table 2 reports the content of each drug and API concentration after sample preparation.

Sample A

1. Grind a tablet of Sample A and transfer into a 50 mL beaker. Add 5 mL CH_3CN and 20 mL mobile phase A. Stir and place in an ultrasonic bath until dissolution is complete. Transfer this solution into a 100 mL volumetric flask, rinse the beaker with mobile phase A a few times, and transfer into the same volumetric flask. Add mobile phase A to bring to volume.
2. Pipet 0.75 mL of this sample solution into a 10 mL volumetric flask and add mobile phase A to bring to volume. Filter with a 0.2 µm filter.

Sample B

1. Prepare a tablet in the same manner as step 1 for Sample A.
2. Pipet 1 mL of this sample solution into a 10 mL volumetric flask and add mobile phase B to bring to volume. Filter with a 0.2 µm filter.

Note: Prepare samples on the day of analysis.

Table 1. Concentrations of Stock and Working Standard Solutions

Compound	Concentration of Stock Standard Solution (mg/L)	Stock Standard Solution Volume (mL)*			Working Standard Solution Concentration (mg/L)		
		L1	L2	L3	L1	L2	L3
Isoniazid	405	1	1.5	2	40.5	60.8	81.0
Pyrazinamide	2198	1	1.5	2	220	330	440
Ethambutol	1452	1	1.5	2	145	218	290
Rifampicin	775	1	1.5	2	78	116	155

*Volume used to prepare 10 mL of working standard solution

Table 2. Tablet Content and Sample Concentration after Sample Preparation

Compound	Sample A		Sample B	
	Tablet Content (mg/tablet)	Calculated Concentration after Sample Preparation (mg/L)	Tablet Content (mg/tablet)	Calculated Concentration after Sample Preparation (mg/L)
Isoniazid	75	56.3	80	80
Pyrazinamide	400	300	250	250
Ethambutol	275	206	—	—
Rifampicin	150	113	120	120

Table 3. Tablet Weights		
Tablet No.	**Tablet Weight (g)**	
	Sample A	**Sample B**
1	1.23	0.71
2	1.19	0.72
3	1.20	0.70
Average	1.21	0.71
Total Weight of APIs (g)	0.91	0.45
Placebo Weight (g)	0.30	0.26

Table 4. Spiked Sample Concentrations				
Compound	**Spiked Standard Amount (mg)**		**Calculated Spiked Concentration after Sample Preparation (mg/L)**	
	In 0.3 g of Sample A Placebo	**In 0.26 g of Sample B Placebo**	**Spiked Sample A Placebo**	**Spiked Sample B Placebo**
Isoniazid	75	80	57	81
Pyrazinamide	400	250	300	250
Ethambutol	275	—	202	—
Rifampicin	150	120	109	116

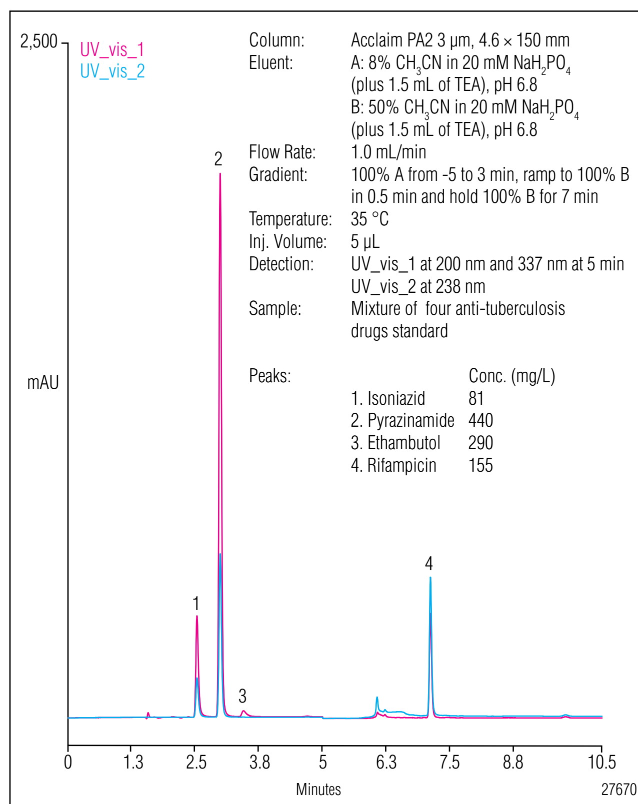

Figure 1. Chromatograms of a standard containing four anti-tuberculosis drugs.

Spiked Placebo Sample

To calculate the placebo weight for each sample, average the weights of the three tablets and subtract the average total API weight of those tablets to obtain the average placebo weight (Table 3). Use the same placebo weight for each sample in Table 3 for the spiked placebo sample preparation. Spike standards (dry) into the placebo to achieve API content similar to the tablet content. Table 4 shows the amount of standards added to each sample placebo and the calculated concentration after sample preparation.

RESULTS AND DISCUSSION

Separation and Detection

The goal of this work was to create one method to determine all four APIs in the combination drug product. The authors started by reviewing the USP monograph for rifampin, isoniazid, pyrazinamide, and ethambutol hydrochloride tablets. The USP monograph has two assay methods. The first assay is for isoniazid, pyrazinamide, and rifampicin. It is an HPLC assay that calls for a 4.6 × 250 mm, 5 µm L1 column, a sodium phosphate buffer pH 6.8/CH_3CN eluent, and a 238 nm detection wavelength. The second assay, which is also an HPLC assay, is for ethambutol. This assay calls for a 4.6 × 150 mm, 5 µm L10 column, triethylamine pH 7/CH_3CN eluent, and a 200 nm detection wavelength. To create a single method for all four APIs, the authors used an Acclaim PA2 (3 µm, 4.6 × 150 mm) column with a sodium phosphate plus triethylamine pH 6.8/CH_3CN eluent. Figure 1 shows a separation of all four compounds in 10 min. The compounds were detected with two UV detection channels. Channel 1 (UV-vis_1) detects compounds by absorbance at 200 nm for the first 5 min and at 337 nm for the final five min. Channel 2 (UV-vis_2) detects at 238 nm. Ethambutol is not detected at 238 nm.

Table 5. Calibration Results at UV-vis_1 as Reported by Chromeleon Software					
Compound	**Cal. Type**	**Points**	**r^2**	**Offset**	**Slope**
Isoniazid	LOff	3	0.99999	-0.3938	0.2920
Pyrazinamide	LOff	3	0.99979	3.1730	0.2684
Ethambutol	LOff	3	0.99938	-0.1861	0.0088
Rifampicin	LOff	3	0.99999	-0.4919	0.1338

Table 6. Calibration Results at UV-vis_2 as Reported by Chromeleon Software					
Compound	**Cal. Type**	**Points**	**r^2**	**Offset**	**Slope**
Isoniazid	LOff	3	0.99994	-0.1826	0.1153
Pyrazinamide	LOff	3	0.99998	-0.4231	0.0812
Ethambutol	—	—	—	—	—
Rifampicin	LOff	3	0.99998	-0.7014	0.1801

Method Calibration

After optimizing sample preparation to determine that all compounds can be detected in each of the two samples, three-point calibration curves were prepared using the two UV channels with the diode array detector. The calibration data in Table 5 show linear peak area response for each detected compound in channel 1 and Table 6 shows linear peak area response for each detected compound in channel 2.

Sample Analysis

Customers provided Samples A and B as well as products without the APIs, referred to as Sample A Placebo and Sample B Placebo. Three tablets for each sample were prepared and three injections of each prepared tablet were made to evaluate the reproducibility of sample preparation, injection, and tablet content. Chromatograms for Samples A and B are shown in Figures 2 and 3, respectively. Sample A contained the expected four APIs, whereas Sample B contained the expected three APIs. Neither tablet contained compounds that interfere with determination of the four APIs. To determine if the four peaks were pure, the photodiode array detector was used for the standard separation. The authors injected single component standards, collected the spectral data, and entered it into the spectral library.

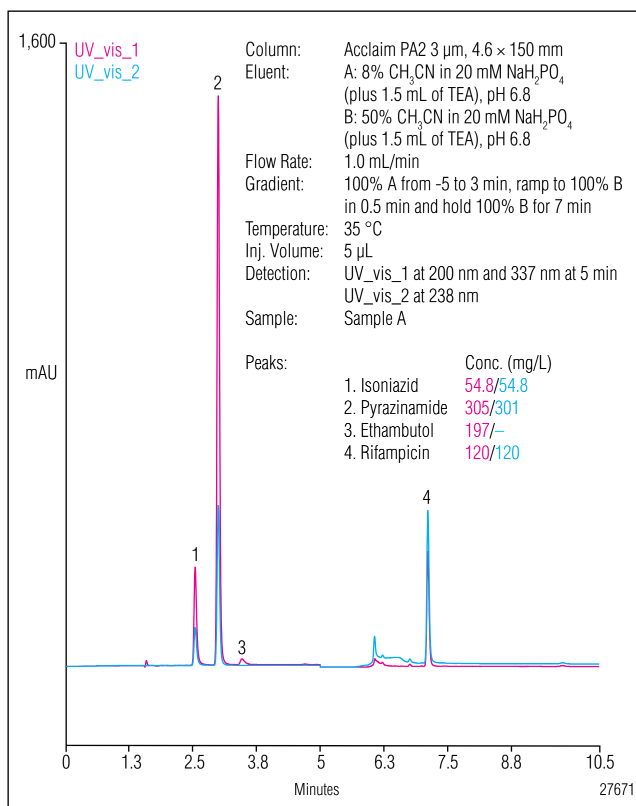

Figure 2. Example chromatograms of Sample A.

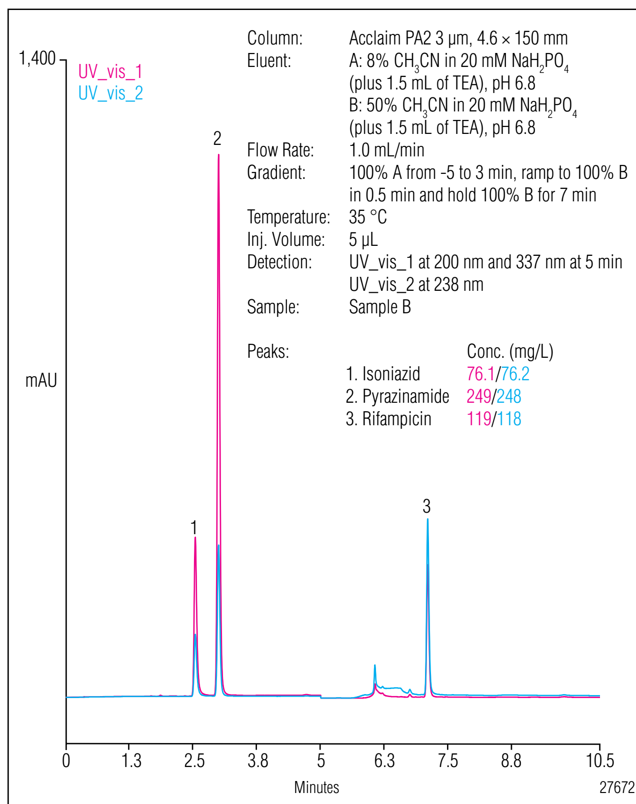

Figure 3. Example chromatograms of Sample B.

Table 7. Peak Purity, Resolution, and Peak Analysis Results for the Standard							
Compound	**Resolution* (USP)**	**Peak Purity Match**	**RSD Peak Purity Match**	**Peak Purity Index**	**RSD Peak Purity Index**	**Asymmetry**	**Plates (USP)**
Isoniazid	4.98	1000	0.47	215.9	0.22	1.42	12410
Pyrazinamide	3.58	1000	0.51	226.2	0.23	1.14	16446
Ethambutol	29.92	995	1.78	194.3	0.80	1.94	7244
Rifampicin	n.a.	998	3.45	284.3	1.21	1.20	—

Table 8. Peak Purity, Resolution, and Peak Analysis Results for the Samples									
Sample	**Compound**	**Peak Purity Match**	**RSD Peak Purity Match**	**Peak Purity Index**	**RSD Peak Purity Index**	**Resolution* (USP)**	**Asymmetry**	**Plates (USP)**	**Match with Library**
Sample A	Isoniazid	1000	0.49	215.9	0.23	4.96	1.38	12185	1000
	Pyrazinamide	1000	0.58	226.2	0.26	3.76	1.16	17480	1000
	Ethambutol	993	2.57	195.0	1.20	30.46	1.81	7705	999
	Rifampicin	997	4.32	284.7	1.50	n.a.	1.16	—	997
Sample B	Isoniazid	1000	0.37	215.9	0.17	4.94	1.37	11999	1000
	Pyrazinamide	1000	0.50	226.3	0.22	47.02	1.14	17053	1000
	Ethambutol	—	—	—	—	—	—	—	—
	Rifampicin	998	2.25	286.0	0.77	n.a.	1.20	—	996

*Calculation is based on USP and is compared to the next main peak.

The data in Table 7 suggest that each peak in the standard was pure. Table 8 shows that all peaks in Samples A and B were pure (judging by the values calculated from the spectral data) and all peaks had very good spectral match with the data entered into library, also suggesting that the peaks in the samples were pure.

Table 9. Average Found Concentration from Three Injections for Three Tablets at UV-vis_1											
Sample	Compound	Calculated Concentration (mg/L)	Tablet 1			Tablet 2			Tablet 3		
			Average	RSD	% Content	Average	RSD	% Content	Average	RSD	% Content
A	Isonaizid	56.3	54.8	0.47	97.34	53.3	0.13	97.26	51.3	0.42	91.12
	Pyrazinamide	300	305	0.69	101.7	303	0.09	101.0	288	0.25	96.00
	Ethambutol	206	197	0.77	95.63	198	0.65	96.12	201	0.65	97.57
	Rifampicin	113	120	0.50	106.2	115	0.28	101.8	120	0.21	106.2
B	Isonaizid	80	76.1	0.08	95.13	76.2	0.16	95.25	73.2	0.42	91.50
	Pyrazinamide	250	249	0.23	99.60	251	0.09	100.4	246	0.37	98.4
	Ethambutol	—	—	—	—	—	—	—	—	—	—
	Rifampicin	120	119	0.15	99.17	125	0.11	104.2	115	0.14	95.83

Table 10. Average Found Concentration from Three Injections for Three Tablets at UV-vis_2											
Sample	Compound	Calculated Concentration (mg/L)	Tablet 1			Tablet 2			Tablet 3		
			Average	RSD	% Content	Average	RSD	% Content	Average	RSD	% Content
A	Isonaizid	56.3	54.8	0.51	97.34	53.2	0.13	94.49	51.4	0.24	91.30
	Pyrazinamide	300	301	0.53	100.3	298	0.10	99.33	284	0.27	94.67
	Ethambutol	206	—	—	—	—	—	—	—	—	—
	Rifampicin	113	120	0.52	106.2	115	0.28	101.8	120	0.18	106.2
B	Isonaizid	80	76.2	0.08	95.25	76.3	0.13	95.38	73.2	0.09	91.5
	Pyrazinamide	250	248	0.15	99.20	249	0.07	99.60	244	0.18	97.6
	Ethambutol	—	—	—	—	—	—	—	—	—	—
	Rifampicin	120	118	0.16	98.33	125	0.12	104.2	115	0.18	95.83

The averaged concentration of APIs in each sample tablet and the RSDs (< 1% for each tablet using both wavelength channels, as shown in Tables 9 and 10) showed good reproducibility of the method and injection. The amounts of each API were compared to the labeled value; for each API for each tablet of Sample A, the amount was between 90 and 110%. The USP monograph for this product specifies that there should be not less than (NLT) 90% and not more than (NMT) 110% of the API in the drug product. The assay demonstrated that each tablet met the USP criteria. The three-API product, Sample B, also passed the NLT 90% and NMT 110% criteria.

To evaluate method accuracy in another manner, the recoveries of APIs added to the sample placebos were determined. Sample placebos without added APIs were also prepared and analyzed with the HPLC method and no interfering compounds were observed (Figure 4). The same amounts of APIs were added to the placebo as shown on the sample label for the drug products. The spiked placebo samples were prepared and three injections were made for each sample. The averaged found concentration in each spiked placebo sample was compared to calculated concentration to determine recovery.

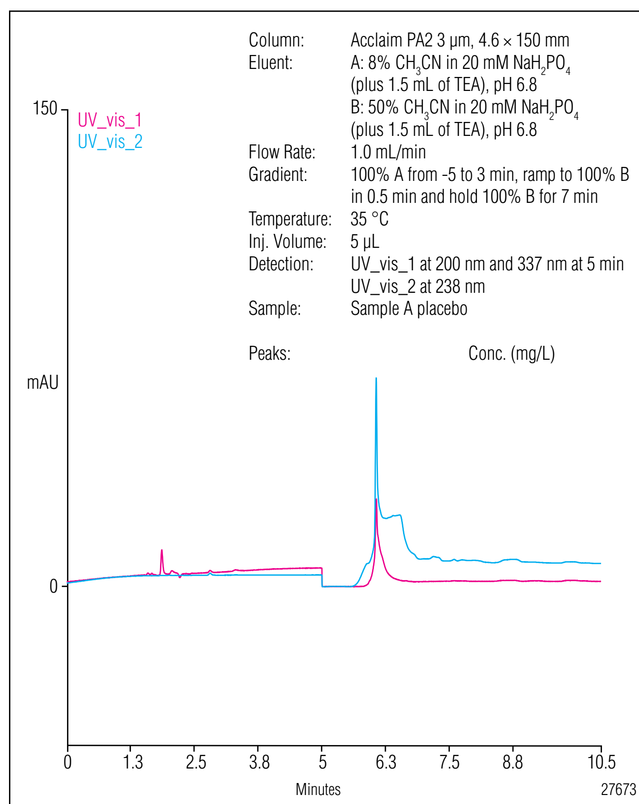

Figure 4. Example chromatograms of Sample A Placebo (chromatograms for Sample B Placebo were equivalent to Sample A Placebo).

Table 11. Recovery at UV-vis_1					
Sample	**Compound**	**Calculated Spiked Concentration (mg/L)**	**Average Found Concentration (mg/L)**	**RSD**	**Recovery**
Spiked Sample A Placebo	Isonaizid	57	55.8	0.06	97.89
	Pyrazinamide	300	299	0.18	99.67
	Ethambutol	202	197	0.17	97.52
	Rifampicin	109	107	0.05	98.17
Spiked Sample B Placebo	Isonaizid	81	78.3	0.11	96.67
	Pyrazinamide	250	250	0.06	100.0
	Ethambutol	—	—	—	—
	Rifampicin	116	110	0.12	94.83

Table 12. Recovery at UV-vis_2					
Sample	**Compound**	**Calculated Spiked Concentration (mg/L)**	**Average Found Concentration (mg/L)**	**RSD**	**Recovery**
Spiked Sample A Placebo	Isonaizid	57	55.8	0.05	97.89
	Pyrazinamide	300	295	0.08	98.33
	Ethambutol	202	—	—	—
	Rifampicin	109	107	0.10	98.17
Spiked Sample B Placebo	Isonaizid	81	78.4	0.06	96.79
	Pyrazinamide	250	248	0.09	99.20
	Ethambutol	—	—	—	—
	Rifampicin	116	109	0.13	93.97

The recoveries in Spiked Placebo Sample A were 97.52 to 99.67% at UV-vis_1 and 97.89 to 98.33% at UV-vis_2, and recoveries in Spiked Placebo Sample B were 94.83 to 100% at UV-vis_1 and 93.97 to 99.20% at UV-vis_2 (Tables 11 and 12). This experiment also indicated that the single HPLC method was accurate for all four APIs.

Faster Analysis

This method can be made faster by using a smaller column format and a smaller particle size. In this work (using the Acclaim RSLC PA2, 2.2 µm, 2.1 × 100 mm column), faster separation was complete in less than 2 min with a system backpressure of ~ 570 bar. Figure 5 shows a chromatogram of faster separation of the four-API standard. Figures 6 and 7 show that faster separation also successfully analyzed the samples.

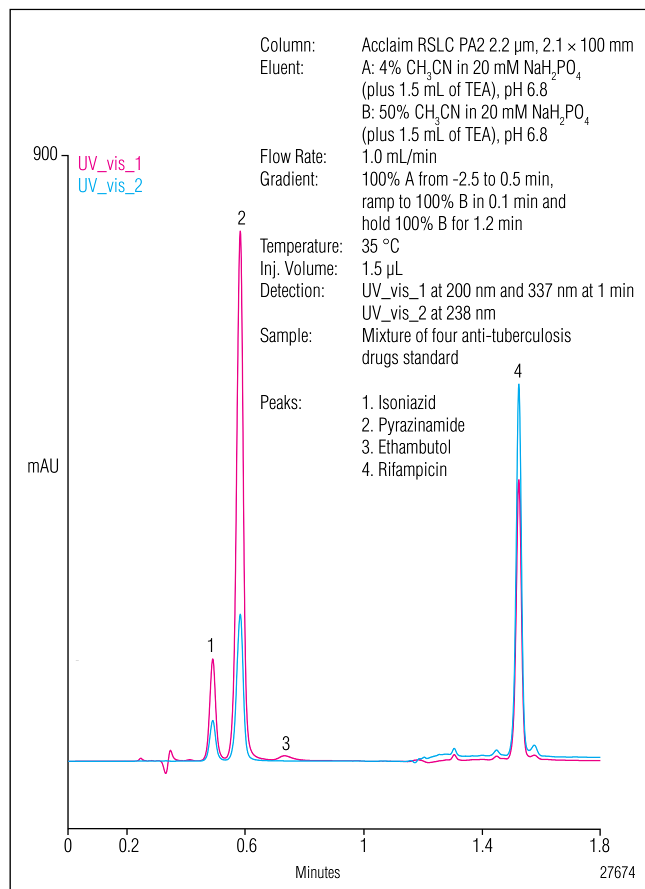

Figure 5. Faster separation of a standard containing four anti-tuberculosis drugs.

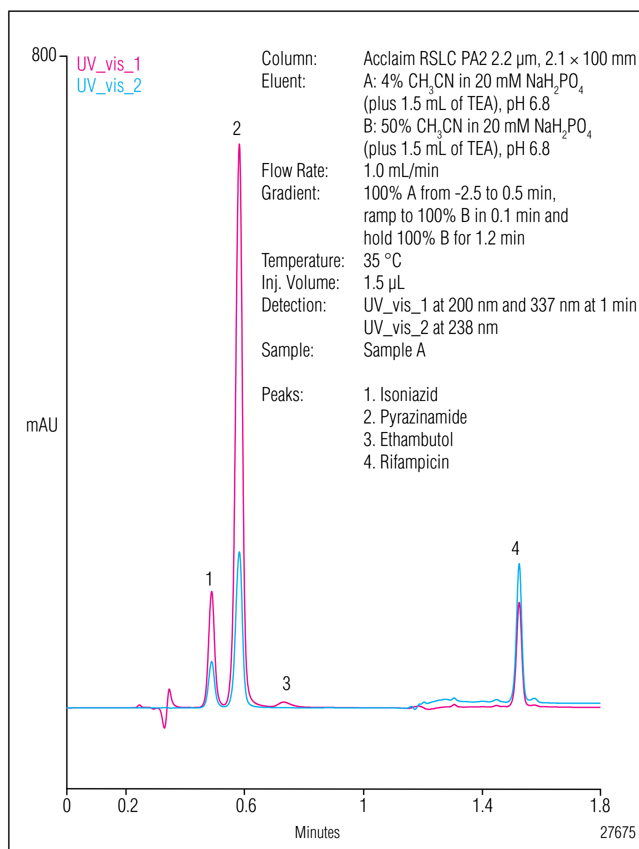

Figure 6. Faster separation of Sample A.

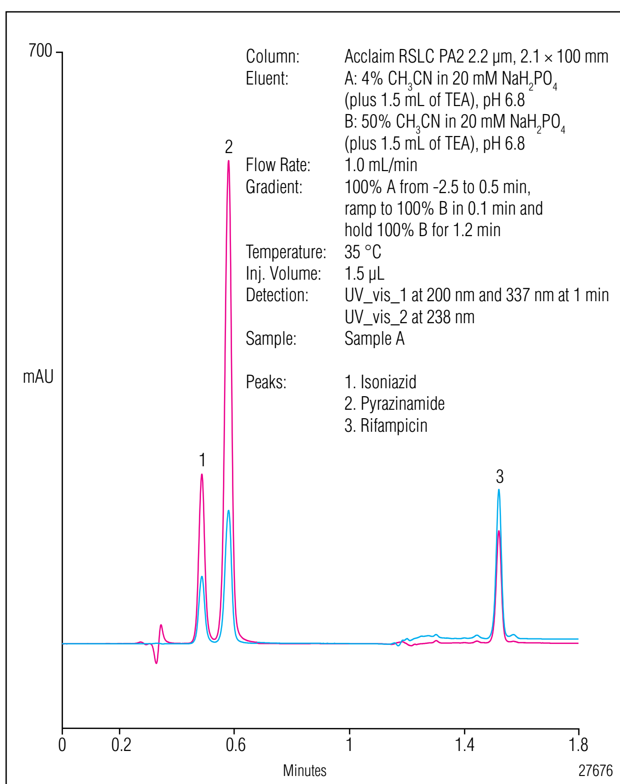

Figure 7. Faster separation of Sample B.

Table 13. Peak Purity, Resolution, and Peak Analysis Results for the Standard (UHPLC)							
Compound	Resolution* (USP)	Peak Purity Match	RSD Peak Purity Match	Peak Purity Index	RSD Peak Purity Index	Asymmetry	Plates (USP)
Isoniazid	2.37	1000	0.12	216.8	0.06	1.00	2775
Pyrazinamide	2.61	1000	0.24	228.5	0.11	0.94	3226
Ethambutol	14.82	989	4.25	196.3	1.97	1.42	1573
Rifampicin	n.a.	1000	0.32	284.9	0.11	0.93	—

Table 14. Peak Purity, Resolution, and Peak Analysis Results for the Samples (UHPLC)									
Sample	Compound	Peak Purity Match	RSD Peak Purity Match	Peak Purity Index	RSD Peak Purity Index	Resolution* (USP)	Asymmetry	Plates (USP)	Match with Library
Sample A	Isoniazid	1000	0.13	216.8	0.06	2.36	1.01	2726	1000
	Pyrazinamide	1000	0.24	228.5	0.11	2.65	0.93	3156	1000
	Ethambutol	989	2.56	195.7	1.02	15.17	1.38	1701	999
	Rifampicin	1000	0.27	284.8	0.09	n.a.	0.93	—	1000
Sample B	Isoniazid	1000	0.13	216.8	0.06	2.33	1.02	2610	1000
	Pyrazinamide	1000	0.25	228.6	0.11	24.79	0.94	3084	1000
	Ethambutol	—	—	—	—	—	—	—	—
	Rifampicin	1000	0.39	285.0	0.14	n.a.	0.93	—	1000

*Calculation is based on USP and is compared to the next main peak.

The peak purity and spectral match data for the standard and samples are displayed in Tables 13 and 14 (spectra from the standard compounds were added to the library for sample analysis) and support the visual observation from Figures 6 and 7 that a successful analysis was achieved with the fast method.

CONCLUSION

This work shows that a single HPLC method can be used to assay the four APIs in a combination drug tablet used to treat TB. This 10 min method saves time as well as mobile phase, compared to the two HPLC assay methods described in the USP monograph for this product. The analysis of two drug products yielded acceptable percentage contents, as judged by the limits in the USP monograph for the four-API drug product. Method accuracy was also tested by spiking standards in the sample placebos and measuring the recovery; there were good recoveries for both samples. The analysis time can be accelerated with the faster method. This separation was complete in < 2 min, providing high throughput sample analysis.

REFERENCES

1. http://en.wikipedia.org/wiki/Tuberculosis_treatment, accessed September 30, 2010.
2. United States Pharmacopeia, 32 NF 27, 2009.

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