High-Throughput LC-MS/MS Measurement of 17-Hydroxyprogesterone in Human Blood Serum for Research Purposes

Joe Di Bussolo¹, Raidiri Castillo², Amit Shah² and Hashim Othman²; ¹Thermo Fisher Scientific, West Chester, PA; ²Bio-Reference Laboratories, Elmwood Park, NJ.

OVERVIEW

Purpose: To develop an LC-MS/MS method for researchers to accurately measure 17-hydroxyprogesterone (17-OHP) in blood serum from 10 to 1,000 ng/dL with a throughput of at least 12 injections per hour and can be multi-channeled with other LC-MS methods utilizing the same ion source.

Methods: Liquid-liquid extraction (LLE) of blood serum followed by quantitative analysis using a 4channel ultra high-performance liquid chromatography (UHPLC) system coupled to a tandem mass spectrometer (MS/MS). 17-OHP and its internal standard were separated from sample matrix components by water-to-methanol gradient elution through a UHPLC column packed with solid-core silica particles having an alkyl-bonded phase on its surfaces. Analytes were eluted to an atmosphericpressure chemical ionization (APCI) probe of a triple-quadrupole mass spectrometer where selectedreaction monitoring (SRM) detected the analytes.

Results: An analytical range of 10 to 1,000 ng/dL was achieved with inter- and intra-batch reproducibility less than 6% CV, carryover less than 0.1% and acceptable correlation of specimen results with those from a reference laboratory. Throughputs of 14 to 56 injections/hour were achieved. Method accuracy and reproducibility were maintained while other channels ran other methods, such as testosterone.

INTRODUCTION

17-OHP is a biosynthetic precursor to other steroids, such as cortisol, androgens and estrogens. It is converted to 11-deoxycortisol by 21α -hydroxylase or to androstenedione by 17, 20 lyase. Researchers investigating how these enzymes function need to measure 17-OHP within an analytical range of 10 to 1,000 ng/dL (0.3 to 30 nmol/L) of blood serum. The chemical structures of 17-OHP and its deuterated internal standard (IS) are shown in Figure 1.

Figure 3. Mass spectrometer APCI & SRM parameters

Ion Source:	APCI, + Ion Current 3 μA, vaporizer temp: 400°C							
SRM Transitions:	Q1 & Q3 resolutions:	0.7						
Analyte	Q1	Q2	CE	RF				
17-OHP (Confirm)	331.20	97.20	22	150				
17-OHP (Quan)	331.20	109.20	26	150				
17-OHP-D ₈ (Confirm)	339.25	100.22	24	160				
17-OHP-D ₈ (Quan)	339.25	113.22	28	160				

RESULTS

Quantitative Analysis Reliability

Typical results from calibrators, QCs and specimens are shown in Figure 4. Ion ratios (Confirming/Quan transitions peak areas) averaged 110% for 17-OHP and 140% for 17-OHP-D8.

IS peak areas among calibrators & QC averaged 8,050 with RSD < 6%. IS peak areas in specimens ranged from 6,670 to 10,290 and showed an average recovery of 102%. Carryover was less than 0.1%. Inter- and intra-batch precisions were less than 5 and 6% coefficient of variation (CV), respectively (Tables 1a & 1b).

Accuracy Assessment

Comparison of 40 specimen results ranging from 11 to 828 ng/dL with reference-lab results showed differences that averaged 3.0% and ranged from -29.6 to 15.4 (Table 2). Only 3 of 40 test samples (7.5%), deviated 20% or more. Deming regression analysis showed $R^2 = 0.9961$, Slope = 0.979, Intercept = 2.8, Standard Error of Estimate = 10.6%.

Table 2. Reference-lab results comparison

Test	Current	Reference	Difference	Test	Current	Reference	Difference
Sample	Method	Lab	%	Sample	Method	Lab	%
1	35	27	-29.6	21	81	71	-14.1
2	37	32	-15.6	22	54	58	6.9
3	28	27	-3.7	23	108	97	-11.3
4	34	27	-25.9	24	112	127	11.8
5	48	48	0.0	25	177	161	-9.9
6	23	23	0.0	26	34	35	2.9
7	22	26	15.4	27	34	31	-9.7
8	18	18	0.0	28	164	168	2.4
9	31	32	3.1	29	37	35	-5.7
10	71	72	1.4	30	91	82	-11.0
11	67	77	13.0	31	94	88	-6.8
12	82	85	3.5	32	207	181	-14.4
13	53	55	3.6	33	53	49	-8.2
14	93	98	5.1	34	63	61	-3.3
15	41	41	0.0	35	84	70	-20.0
16	44	43	-2.3	36	13	11	-18.2
17	46	48	4.2	37	80	85	5.9
18	121	114	-6.1	38	246	213	-15.5
19	42	39	-7.7	39	185	199	7.0
20	25	24	-4.2	40	12	11	-9.1

Figure 1. Chemical structures of analyte and IS





17α-Hydroxyprogesterone (17-OHP) internal standard Formula: $C_{21}H_{30}O_3$ Mono-isotopic mass: 330.2189

17α-Hydroxyprogesterone-D₈ (**17-OHP-D**₈) internal standard Formula: $C_{21}H_{22}D_8O_3$ Mono-isotopic mass: 338.2692

MATERIALS AND METHODS

Consumables

Fisher Scientific[™] Optima[™] solvents were used for LC mobile phases and wash solutions, as well as for preparations of calibrators, quality controls (QCs) and donor blood serum samples. 17α-Hydroxyprogesterone and 17α-Hydroxyprogesterone-D8 were purchased from Cerilliant Corporation (Round Rock, TX). Calibrators were made by mixing stock standard with a diluent of 1% bovine serum albumin in phosphate-buffered saline. 17-OHP controls were purchased from UTAK Laboratories Inc. (Valencia, CA).

Sample Preparation

200 µL aliquots of fresh blood serum specimens, as well as calibrators and quality control specimens (QCs), were spiked with 17-OHP-D8 internal standard (IS) before being subjected to liquid-liquid extraction with 1 mL methyl t-butyl ether (MTBE). After drying the ether extracts by heated nitrogen flow, the residue of each tube was reconstituted with water and methanol (1:1) to a total volume of 150 µL, and 50 μL injections were made into the LC-MS/MS system.

Throughput

Single-channel throughput was 14 injections per hour. When multi-channeled across 2, 3 or 4 channels, the throughput increased to 28, 42 and 56 injections per hour, respectively. 17-OHP batches were also multi-channeled with MMA (1) batches, which utilized the same MS source conditions.

Figure 4. Typical 17-OHP quantitative results

		Com	ounds		→ ₽ ×	Sam	iple Res	ults										↓ 1	×
As As <td< th=""><th></th><th>1</th><th>Compound</th><th>Compound Type</th><th>Expected RT</th><th>₽</th><th>Flags</th><th>Status</th><th>Level</th><th>Sample Name</th><th>Area</th><th>Actual RT</th><th>Calculated</th><th>Final Units</th><th>Theoretical</th><th>%Diff</th><th>ISTD Resp(</th><th>Excluded</th><th>-</th></td<>		1	Compound	Compound Type	Expected RT	₽	Flags	Status	Level	Sample Name	Area	Actual RT	Calculated	Final Units	Theoretical	%Diff	ISTD Resp(Excluded	-
1 12-OH-P Target Compound 0.40 2 15 Internal Standard 0.40 2 15 Internal Standard 0.40 4 Cal C 2 133 ng/dt 11.000 3.21 7724 5 Cal 3 Cal 2 181 1323 ng/dt 11000 3.21 7724 1 5 Cal 3 Cal 3 2595 0.43 97.532 ng/dt 110000 -11.33 7567 6 Cal 4 Cal 5 15785 0.43 255.22 ng/dt 25000 0.42 8.40 1 7 Cal 5 Cal 5 15785 0.43 255.22 ng/dt 110000 -11.33 7687 8 Cal 6 Cal 4 Cal 4 783 0.43 250.23 ng/dt N/A N/A 100 9 Cal 6 0.43 30.631 ng/dt N/A N/A 101 0 0.22 24.35 0.43 90.631 ng/dt N/A N/A 101 101 0 0.22 101 N/A N/A<			<u>A</u> a ▼	<u>A</u> a 🔻	<u>A</u> a ▼			<u>A</u> a ·	<u>A</u> a ·	<u>A</u> a ▼	<u>A</u> a ·	<u>A</u> a 🔻	<u>A</u> a ▼	<u>A</u> a 🔻	<u>A</u> a ▼	<u>A</u> a •	<u>A</u> a ▼		
2 15 Internal Standard 0.40 3 Cal Cal 2 135 ng/dL 11.00 3.21 77.4 = 4 Cal 2 135 0.41 58.751 ng/dL 55.000 6.82 8651 5 Cal Cal 2 100 0.1133 7577 0.62 6 Cal Cal Cal 2 10.000 1.133 7577 0.62 6 Cal Cal Cal 5 10.000 0.42 812 10.000 10.000 10.000 10.000 0.42 812 10.000 10.000 10.000 0.42 812 10.000 10.000 10.000 0.42 812 10.000 10.000 0.42 10.000 0.42 10.000 10.000 0.42 10.000 10.000 0.42 10.000 0.42 10.000 0.42 10.000 0.41 10.0000 0.41 10.0000 0.01 10.000 0.01 10.000 0.01 10.000 0.01 10.000 0.01 10.0000		1	17-OH-P	Target Compound	0.40	2	1	•		Cal 0-Pre	N/F	N/F	N/F	ng/dL	N/A	N/A	9592		
4 Cal 2 Cal 2 1831 0.41 58.751 ng/dL 55.000 6.62 8651 5 Cal 3 Cal 3 2555 0.43 97.352 ng/dL 110.000 -11.13 7577 6 Cal 4 Cal 4 Cal 5 15785 0.43 97.352 ng/dL 275.000 0.42 8192 7 Cal 5 Cal 5 15785 0.43 552.322 ng/dL N/A N/A 98 9 Cal 6 Cal 6 Cal 6 30087 0.41 104.807 ng/dL N/A N/A 98 0.210 ng/dL N/A N/A 99 0.43 30.631 ng/dL N/A N/A 913 1 1 0 5 5 1 1 0.44 35.637 ng/dL N/A N/A 9.43 30.631 ng/dL N/A N/A 1 1 1		2	IS	Internal Standard	0.40	3		•	Cal 1	Cal 1	322	0.43	11.353	ng/dL	11.000	3.21	7724		-
5 Cal 3 Cal 3 2595 0.43 97.532 ng/dL 110.000 -11.33 7587 6 Cal 4 Cal 5 Cal 5 Cal 5 15785 0.43 552.322 ng/dL 275.000 0.45 8140 7 Cal 5 Cal 5 Cal 5 Cal 5 Cal 6 10080 0.41 110.4000 ng/dL N/A N/A 9831 10 QC 1 8876 0.41 328.616 ng/dL N/A N/A 9831 11 QC 2 Specimen 1 3358 0.41 104.899 ng/dL N/A N/A 913 12 Specimen 2 993 0.43 30.631 ng/dL N/A N/A 913 13 Specimen 3 500 0.42 356.37 ng/dL N/A N/A 917 14 Specimen 5 1007 0.41 39.126 ng/dL N/A N/A 917 1 14 Specimen 5 1007 0.41 39.126 ng/dL N/A N/A 7 7						4		•	Cal 2	Cal 2	1831	0.41	58.751	ng/dL	55.000	6.82	8851		
6 Cal 4 Cal 4 7853 0.42 276.236 ng/dL 275.000 0.45 8140 7 Cal 5 Cal 5 Cal 5 Cal 5 Cal 5 104.807 ng/dL 100.000 0.44 8192 9 Cal 6 Cal 6 Cal 6 Cal 6 0.43 522.32 ng/dL N/A N/A 8140 9 Cal 6 Cal 6 Cal 6 Cal 6 0.43 50.000 0.42 8192 10 QC1 8876 0.41 328.616 ng/dL N/A N/A 933 11 QC2 24376 0.42 919.155 ng/dL N/A N/A 9133 12 Specimen 1 358 0.41 104.899 ng/dL N/A N/A 9133 13 Specimen 2 993 0.43 30.631 ng/dL N/A N/A 917 14 Specimen 3 500 0.42 35.637 ng/dL N/A N/A 917 15 Specimen 4 288.0 0.43 90.492 <th></th> <th></th> <th></th> <th></th> <th></th> <th>5</th> <th></th> <th>•</th> <th>Cal 3</th> <th>Cal 3</th> <th>2595</th> <th>0.43</th> <th>97.532</th> <th>ng/dL</th> <th>110.000</th> <th>-11.33</th> <th>7587</th> <th></th> <th></th>						5		•	Cal 3	Cal 3	2595	0.43	97.532	ng/dL	110.000	-11.33	7587		
7 Cal 5 Cal 5 15785 0.43 552.322 ng/dL 550.000 0.42 8192 8 Cal 6 Cal 6 Cal 70st 28 0.38 0.210 ng/dL N/A N/A N/A 9831 9 Cal 0-Post 28 0.38 0.210 ng/dL N/A N/A N/A N/A 9831 10 QC1 8876 0.41 328.616 ng/dL N/A N/A N/A N/A N/A 9133 11 QC2 24376 0.42 919.155 ng/dL N/A N/A N/A 9133 12 Specimen 1 3358 0.41 104.899 ng/dL N/A N/A 9137 1 14 Specimen 3 850 0.42 35.637 ng/dL N/A N/A 9397 1 14 Specimen 5 1007 0.41 39.042 ng/dL N/A N/A 9397 1 15 Specimen 5 1007 0.41 39.126 ng/dL N/A N/A						6		•	Cal 4	Cal 4	7853	0.42	276.236	ng/dL	275.000	0.45	8140		L
8 Cal 6 Cal 6 30067 0.41 1104.807 ng/dL 1100.000 0.44 7810 9 0 Cal 6 Cal 6 0.087 0.41 1104.807 ng/dL N/A N/A N/A 9831 10 QC1 8876 0.41 328.616 ng/dL N/A N/A N/A 9931 11 QC2 24376 0.42 919.155 ng/dL N/A N/A 7737 12 Specimen 1 3358 0.41 104.899 ng/dL N/A N/A 9133 13 Specimen 2 993 0.43 30.631 ng/dL N/A N/A 9137 14 Specimen 3 850 0.42 35.637 ng/dL N/A N/A 9733 16 Specimen 5 1007 0.41 39.126 ng/dL N/A N/A 7737 14 Specimen 5 1007 0.41 39.126 ng/dL N/A N/A 7737 15 Str.5318 1/1 Str.5318 3.6						7		•	Cal 5	Cal 5	15785	0.43	552.322	ng/dL	550.000	0.42	8192		
9 Cal 0-Post 28 0.38 0.210 ng/dL N/A N/A 9831 10 QC 1 8876 0.41 328.616 ng/dL N/A 9133 13 Specimen 3 850 0.42 95.637 ng/dL N/A N/A 9133 14 Specimen 4 2983 0.43 90.492 ng/dL N/A N/A 9391 Specime 1 30.631 ng/dL N/A N/A 9391 N/A N/A 9391 Specime 1 30.631 ng/dL N/A N/A 9275 N/A N/A Specime 1 Specime 1 Specimen 5 100 <th></th> <th></th> <th></th> <th></th> <th></th> <th>8</th> <th></th> <th>•</th> <th>Cal 6</th> <th>Cal 6</th> <th>30087</th> <th>0.41</th> <th>1104.807</th> <th>ng/dL</th> <th>1100.000</th> <th>0.44</th> <th>7810</th> <th></th> <th></th>						8		•	Cal 6	Cal 6	30087	0.41	1104.807	ng/dL	1100.000	0.44	7810		
10 QC 1 8876 0.41 328.616 ng/dL N/A N/A N/A 7737 11 QC 2 24376 0.42 919.155 ng/dL N/A N/A N/A 10 12 Specimen 1 3358 0.41 104.899 ng/dL N/A N/A 9133 13 Specimen 2 993 0.43 30.631 ng/dL N/A N/A 9133 14 Specimen 3 850 0.42 35.637 ng/dL N/A N/A 9137 15 Specimen 4 2983 0.43 90.492 ng/dL N/A N/A 917 14 Specimen 5 1007 0.41 39.126 ng/dL N/A N/A 9397 S Compound Details Confirming Ions Calibration Curve Y S						9		•		Cal 0-Post	28	0.38	0.210	ng/dL	N/A	N/A	9831		
11 QC 2 24376 0.42 919.155 ng/dL N/A N/A N/A 913.15 12 Specimen 1 3358 0.41 104.899 ng/dL N/A N/A 913.15 13 Specimen 2 993 0.43 30.631 ng/dL N/A N/A 913.15 14 Specimen 3 850 0.42 35.537 ng/dL N/A N/A 6733 15 Specimen 5 1007 0.41 39.126 ng/dL N/A N/A 6733 16 Specimen 5 1007 0.41 39.126 ng/dL N/A N/A 7775 F Quan Peak Stasses 1/1 N/A						10		•		QC 1	8876	0.41	328.616	ng/dL	N/A	N/A	7737		
12 Specimen 1 3358 0.41 104.899 ng/dL N/A N/A 9133 13 Specimen 2 993 0.43 30.631 ng/dL N/A N/A 9117 14 Specimen 3 850 0.42 35.637 ng/dL N/A N/A 937 15 Specimen 4 2983 0.43 90.492 ng/dL N/A N/A 937 16 Specimen 5 1007 0.41 39.126 ng/dL N/A N/A 937 Compound Details Image: Confirming Lons Image: Confirming						11		•		QC 2	24376	0.42	919.155	ng/dL	N/A	N/A	7605		
13 Specimen 2 993 0.43 30.631 ng/dL N/A N/A 9117 14 Specimen 3 850 0.42 35.637 ng/dL N/A N/A 6733 15 Specimen 4 2983 0.43 90.492 ng/dL N/A N/A 6733 15 Specimen 5 1007 0.41 39.126 ng/dL N/A N/A 9397 Compound Details Confirming Ions V X Calibration Curve V V 7.0HP 17-0H-P RT: 0.43 8293555 1/1 RT: 0.43 8293555 X Calibration Curve V 3.485e-3X + 2.122e-3; R^2: 0.9991; Ongin: Ignore; W: 1/X; Area Ad: 802.68						12	-	•		Specimen 1	3358	0.41	104.899	ng/dL	N/A	N/A	9133		
14 Specimen 3 850 0.42 35.637 ng/dL N/A N/A 6733 15 Specimen 4 2983 0.43 90.492 ng/dL N/A N/A 9397 16 Specimen 5 1007 0.41 39.126 ng/dL N/A N/A N/A 9397 Quan Peak Image: Confirming Ions						13		•		Specimen 2	993	0.43	30.631	ng/dL	N/A	N/A	9117		
15 Specimen 4 2983 0.43 90.492 ng/dL N/A N/A 9397 Compound Details						14		•		Specimen 3	850	0.42	35.637	ng/dL	N/A	N/A	6733		
Image: Market Bill Is Specimen 5 1007 0.41 39.126 ng/dL N/A N/A 7275 Compound Details Image: Market Bill						15		•		Specimen 4	2983	0.43	90.492	ng/dL	N/A	N/A	9397		
Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Quan Peak Image: Compound Details Image: Compound Details Image: Compound Details Quan Peak Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Quan Peak Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Quan Peak Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details Image: Compound Details		4			Þ	16	-	•		Specimen 5	1007	0.41	39.126	ng/dL	N/A	N/A	7275		
Quan Peak Confirming Ions Calibration Curve 17-OH-P N: 0.43 8293555 1/1 RT: 0.43 829355 1/1 RT: 0.43 82935 1/1 RT: 0.		Com	ound Detail	s														ب ب مار	X
17-OH-P RT: 0.43 8293555 1/1 RT: 0.43 8293555 Y = 3.485e-3X + 2.122e-3; R^2: 0.9991; Origin: Ignore; W: 1/X; Area 100 RT: 0.43 8293555 RT: 0.43 8293555 A: 1005 28 A: 307.83 100 SN: 331.08 Interpret of the second seco	L	Quar	Peak	_	T	Confi	irming I	ons 🗨	-	▼ ×	Calibr	ation Curve	•					Ţ ×]
FT: 0.43 AA: 902.56 AH: 384.03 SN: 331.08 FT: 0.43 AA: 1005.28 AH: 377.52 4.0 100 90 80 80 70 100 90 80 80 70 100 90 80 80 70 100 90 80 80 70 100 90 80 80 70 100 90 80 80 80 80 80 80 80 80 80 80 80 80 80		17-	OH-P RT: 0.43	8293555	1/1	RT	: 0.43 8;	293555				Y	= 3.485e-3X +	1 2.122e-3; R^2	7-OH-P 2: 0.9991; Origir	n: Ignore; \	N: 1/X; Area		
		Relative Intensity	100 90 80 70 60 50 40 20	RT: 0.43 AA: 992.56 AH: 384.03 SN: 331.08		Relative Intensity	100 90 80 70 60 50 40 30 20		RT: 0.43 AA: 1005.28 AH: 367.63 SN: 377.52		4.0 3.5 3.0 2.5 4.0 3.0 2.0 1.5				_				

CONCLUSIONS

Robust, reliable and sensitive quantification of 17-OHP in donor blood serum samples was achieved using this research method with a four-channel LC-MS/MS system. We demonstrated:

- Analytical range from 10 to 1,000 ng/dL
- Throughputs of 14, 28 or 56 injections per hour from a 1-, 2- or 4-channel system
- Inter- & intra-batch precisions less than 6% and carryover less than 0.1%
- Multi-channeling with other methods utilizing the same APCI source

ACKNOWLEDGEMENTS

The authors thank Ian White and Emily Herman who contributed to this work while serving as interns of the Pharmaceutical Product Development Program of West Chester University of Pennsylvania.

TRADEMARKS/LICENSING

© 2019 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is not intended to encourage use of these products in any manner that might infringe the intellectual property rights of others.

For research use only. Not for use in diagnostic procedures.

Test Methods

Using one or more channels of a Thermo Scientific[™] Transcend[™] LX-4 system, chromatographic segregation of the steroids from unwanted sample components was accomplished by gradient elution through a Thermo Scientific[™] Accucore[™] RP-MS column (2.6 µm, 50 x 2.1 mm), which was heated to 50°C. Chromatographic conditions are described in Figure 2. The Thermo Scientific[™] TSQ Endura[™] triple-quadrupole mass spectrometer was used with an APCI probe. Ion source and MS/MS conditions are described in Figure 3.

Instrument Control & Data Analysis

Thermo Scientific[™] TraceFinder[™] software with Aria[™] MX software was used to control the Transcend LX-4 and Endura MS/MS systems, submit batches to desired channels, as well as for analyzing data and reporting results.

Figure 2. Liquid chromatography parameters

	Column:	Accucore	RP-MS, 2	.6 μ, 50 x 2.1	mm	
	Solvent A:	Water + 0	.1% Formi	c Acid		
	Solvent B:	Methanol				
Step	Start	Sec	Flow	Gradient	%A	% E
1	0.00	10	0.5	Step	90	10
2	0.17	20	0.5	Step	50	50
3	0.50	60	0.5	Ramp	30	70
4	1.50	30	0.5	Ramp	-	100
5	2.00	30	0.5	Step	-	100
6	2.50	30	0.5	Step	90	10
7	2 00	60	07	Stop	00	10



Table 1. In	ter- and intra-batch a. Inter-batch	pr	ecision results	n results b. Intra-batch			
Injection	17-OHP (ng/dL)		Date	Injection	17-OHP (ng/dL)		
1	86		Day 1, 10/7/14	1	86		
2	74			2	74		
3	80			3	80		
4	85			4	85		
5	84			5	84		
6	84		Day 2, 10/8/14	1	80		
7	84			2	77		
8	79			3	78		
9	83			4	82		
10	76			5	89		
11	75		Day 3, 10/9/14	1	85		
12	86			2	85		
13	81			3	76		
14	78			4	77		
15	83			5	87		
16	83		Day 4, 10/10/14	1	77		
17	87			2	82		
18	83			3	74		
19	84			4	86		



20	86		5	77
Mean	82.05		Mean	81.05
STD	3.85		SD	4.63
CV %	4.7		CV %	5.71
a. Inter-b	batch	b. In	tra-batch	

PO73136-EN 0719S

