Pharmaceuticals in Whole Blood Analyzed on Agilent Poroshell 120, with Modified Mini-Extraction for Sample Preparation

Application Note

Biopharmaceuticals

Author

Joan Stevens
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA

Abstract

A convenient analytical method for determination of pharmaceuticals in various therapeutic categories in whole blood involves the addition of acetonitrile and salts to a small amount of blood. The mixture is shaken and centrifuged for extraction/partitioning, which removes water and proteins from the sample. An aliquot of the organic layer is cleaned by dispersive solid-phase extraction (SPE) employing SPE sorbent and salts, to remove endogenous matrix components. Analytes are then isolated from spiked samples with recoveries above 80% on average, and RSDs typically below 10% for a wide range of substances. This mini-extraction approach in whole blood delivers successful separation for a variety of pharmaceuticals, with limits of detection below 10 ng/mL. The method is quick, easy, inexpensive, and effective for therapeutic drug monitoring and forensic chemistry.

Agilent Technologies

Introduction

Determination of pharmaceuticals in biological matrices is commonly employed in ADME (DMPK), clinical and forensic analysis. The main techniques used for drug monitoring and analysis are immunoassays, LC, and GC methods. Mass spectral chromatographic methods are the first choice for many applications based on their flexibility, selectivity, sensitivity, qualitative and quantitative capabilities. Analysis of pharmaceuticals in biological samples requires sample preparation that can range from simple protein precipitation (PPT) to more complex solid-phase extraction (SPE). There is a need in classic sample preparation for a method to determine multi-classes of pharmaceuticals in biological samples. Polymeric or mixed-mode SPE sorbents that can isolate acidic, neutral and basic drugs by hydrophobic and, or ion-exchange interactions address this need but there is always room for sample preparation techniques that are rapid and inexpensive to implement.

Previously reported methods provide analysis of multi-residue pesticides in foods. They are known as QuEChERS, (a quick, easy, cheap, effective, rugged, and safe sample preparation approach) [1]. The authors reported outstanding recoveries for a wide range of pesticide classes. Since its inception there have been many reported articles employing QuEChERS for the analysis of a wide range of compounds including, but not specific to, antibiotics [2], toxins [3], contaminants [4], and pharmaceuticals [5].

In this note we describe an extension of the work presented by Plössl et al. in 2006, for the determination of pharmaceuticals in whole blood employing a modified mini-extraction procedure with LC-MS/MS analysis. The experiments presented in this application note used human whole blood containing either EDTA or citrate as an anticoagulant and were evaluated with both non-buffered and buffered extraction salts used in the QuEChERS methodology, namely non-buffered, AOAC 2007.01 and EN 15662. Modifications to the acetonitrile (extraction solvent) used in the first step (extraction/partitioning) were also evaluated. The experiments were performed using nine different pharmaceuticals (lidocaine, tramadol, amitriptyline, biperidene, oxazepam, lorazepam, chlorpromazine, diltiazem, and naloxone), with a broad range of hydrophobicity and dissociation constants (Table 1). Agilent Poroshell 120 is a good column for this analysis, in part because it has standard 2-µm frits and is more forgiving for more complex samples relative to a sub-2-µm column. Poroshell 120 has mass transfer such that it acts very much like a sub-2-µm particle LC column, without the high back pressure associated with a sub-2-µm column. The efficient mass transfer equates with faster analysis time and higher throughput with optimum resolution.

Table1. Characteristics of Pharmaceuticals Under Investigation

Compound	CAS number	Log P	pKa	Therapeutic use
Lidocaine	*137-58-6*	*2.4*	*8.01*	*Local anesthetic, antiarrhythmic*
Tramadol	*27203-92-5*	*2.5*	*9.41*	*Analgesic*
Amitriptyline	*50-48-6*	*4.92*	*9.4*	*Antidepressant*
Biperidene	*514-65-8*	*4.0*	*10.8*	*Anticholinergic*
Oxazepam	*604-75-1*	*2.23*	*1.7, 11.3*	*Antianxiety*
Lorazepam	*846-49-1*	*2.47*	*1.3, 11.5*	*Antidepressant*
Chlorpromazine	*50-53-3*	*5.18*	*9.3*	*Antipsychotic*
Diltiazem	*42399-41-7*	*3.63*	*7.7*	*Calcium channel blocker*
Naloxone	*465-65-6*	*1.45*	*7.9*	*Opioid receptor antagonist*
Nortriptyline (IS)	*72-69-5*	*5.65*	*9.7*	

Experimental

All reagents and solvents were HPLC analytical grade. The compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA).

A stock solution of 1 M ammonium acetate (NH_4OAc) pH 5 was made by dissolving 19.27 g of NH_4OAc powder in 250 mL Milli-Q water. The pH was adjusted to 5 with acetic acid monitored with a pH meter. The solution was stored at 4 °C. MeOH:H_2O (20:80) containing 5 mM NH_4OAc pH 5 was made by combining 200 mL MeOH and 800 mL Milli-Q water, adding 5 mL of 1 M NH_4OAc, pH 5 stock solution. The 5 mM NH_4OAc in ACN was prepared by adding 5 mL of 1 M NH_4OAc, pH 5 stock solution to 1L ACN, sonicating well.

Standard and internal standard solutions (2.0 mg/mL) were made in MeOH and stored at –20 °C. A QC spiking solution of 5.0 µg/mL was made fresh daily in 1:1 ACN:H_2O (0.1% FA). A 0.5 and 5.0 µg/mL standard solution in 1:1 ACN:H_2O (0.1% FA) was made for the preparation of calibration curves in the matrix blank extract with appropriate dilution. A 5 µg/mL of nortriptyline in 1:1 ACN:H_2O (0.1% FA) was used as the internal standard (IS).

Equipment

- Agilent 1260 HPLC with Diode Array
- Agilent 6460 Triple Quadrupole LC/MS system with Electrospray Ionization
- Agilent Bond Elut QuEChERS AOAC Extraction kit (p/n 5982-6755)
- Bond Elut QuEChERS EN Extraction kit (p/n 5982-6650)
- Bond Elut QuEChERS Non-Buffered Extraction kit (p/n 5982-6550)
- Bond Elut QuEChERS AOAC Dispersive SPE kit for General Fruits and Vegetables (p/n 5982-5022)
- Bond Elut QuEChERS EN Dispersive SPE kit for General Fruits and Vegetables (p/n 5982-5021)
- Bond Elut Ceramic Homogenizers (p/n 5982-9312)
- Sorvall ST 16R Centrifuge (Thermo IEC, MA, USA)
- Micro centrifuge 5415D Eppendorf (Brinkman Instruments, Westbury, NY, USA)
- Geno Grinder 2010 (SPEX CertiPrep, Inc., Metuchen, NJ, USA)
- DVX 2500 Multi-Tube Vortexer (VWR International, West Chester, PA, USA)

HPLC conditions

Column	Agilent Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-902)
Flow rate	0.4 mL/min
Column temperature	30 °C
Injection	10 µL
Mobile phase	A. 5 mM Ammonium acetate, pH 5 in 20:80 MeOH:water
	B. 5 mM Ammonium acetate, pH 5 in ACN
Needle wash	1:1:1:1 ACN:MeOH:IPA:H_2O (0.2% FA)
Gradient	20 to 75% B over 5.5 min

MS conditions

ESI	Positive mode
GT	300 °C
GF	7 L/min
Nebulizer	40 psi
SGT	400 °C
SFG	12 L/min
Capillary	3500 V
NV	500 V

Other MS conditions relating to the analytes are listed in Table 2.

Table 2. Instrument Acquisition Data Used for the Analysis of Nine Drugs by LC-MS/MS

Compound	MRM channels (m/z)	Fragmentor (V)	CE (V)	RT (min)	Delta RT
Lidocaine	1) 235.18 > 86.1 2) 235.18 > 58.1	97	11 35	1.37	0.4
Tramadol	1) 264.2 > 58.1 2) 264.2 > 246.1	97	15 3	1.20	0.4
Amitriptyline	1) 278.2 > 117 2) 278.2 > 105	112	19 19	4.25	0.4
Biperidene	1) 312.23 > 98.1 2) 312.23 > 55.1	123	19 60	4.23	0.7
Oxazepam	1) 287.06 > 240.9 2) 287.06 > 268.9	112	19 7	3.99	0.4
Lorazepam	1) 321.02 > 274.9 2) 321.02 > 302.9	113	15 7	4.09	0.4
Chlorpromazine	1) 319.11 > 86.1 2) 319.11 > 58.1	112	15 43	4.63	0.4
Diltiazem	1) 415.17 > 177.9 2) 415.17 > 149.9	128	19 43	3.73	0.4
Naloxone	1) 328.16 > 310 2) 328.16 > 212	123	15 39	0.82	0.4
Nortriptyline (IS)	1) 264.18 > 233 2) 264.18 > 91	97	7 19	4.17	0.4

General procedure

1. Add 1 mL of whole blood to a centrifuge tube.
2. Spike with appropriate volume from a concentrated stock mixture to yield 25, 50, and 100 ng/mL of the component mix.
3. Add 20 µL of IS stock solution, yield 100 ng/mL (Nortriptyline), and two ceramic homogenizers.
4. Vortex.
5. Add 2 mL of an acetonitrile solution (with or without acid), see Table 3.
6. Vortex.
7. Add a premixed amount (see Table 3) of the extraction salts and vigorously shake.
8. Centrifuge at 5,000 rpm for 5 minutes.
9. Transfer 1 mL of the extract into a d-SPE tube (2 mL centrifuge tube) containing 50 mg of PSA and 150 mg of $MgSO_4$ for matrix clean-up.
10. Vortex for 1 minute.
11. Centrifuge at 18,000 rpm for 3 minutes.
12. Transfer a 200 µL aliquot of the extract into a LC vial containing 800 µL of water.
13. Vortex and analyze.

The entire series of experiments that were evaluated can be found in Table 3. A matrix matched calibration curve from 10-250 ng/mL was employed to determine recovery.

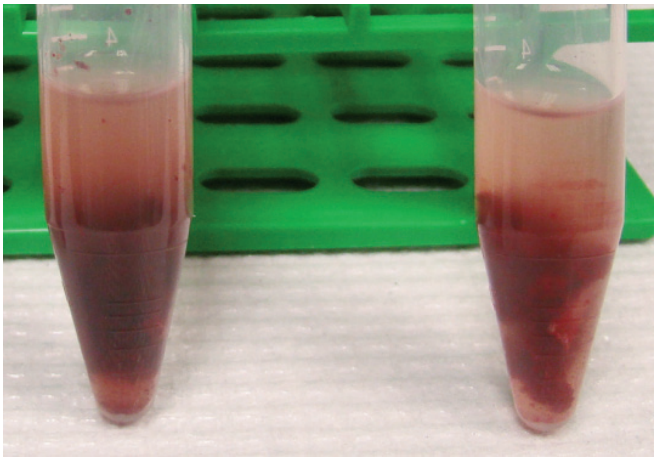
Table 3. Series of Experimental Conditions Investigated

Sample (1 mL)	Extraction solvent	Extraction salts (mg)	d-SPE	Observation
WB	ACN	none	none	Sample: solid mass
WB	ACN, 1% AA	none	none	Sample: solid mass
WB	ACN, 0.4% FA	none	none	Sample: loose particles
WB	ACN, 0.4% FA	Non-buffered, 500	none	Dark extract
WB	ACN, 0.4% FA	AOAC, 500	none	Clear extract
WB	ACN, 0.4% FA	EN, 650	none	Dark extract
WB	ACN, 0.4% FA	Non-buffered, 500	50 mg PSA, 150 mg $MgSO_4$	Clear extract
WB	ACN, 0.4% FA	AOAC, 500	50 mg PSA, 150 mg $MgSO_4$	Clear extract
WB	ACN, 0.4% FA	EN, 650	25 mg PSA, 150 mg $MgSO_4$	Clear extract
WB	ACN, 0.4% FA	EN, 650	50 mg PSA, 150 mg $MgSO_4$	Clear extract

WB = whole blood; ACN = Acetonitrile, AA = acetic acid; FA = formic acid,
PSA = Primary secondary amine, AOAC = $MgSO_4$ & NaAcetate, EN= $MgSO_4$ & Citrate buffers,
Non-buffered = $MgSO_4$ & Sodium Chloride

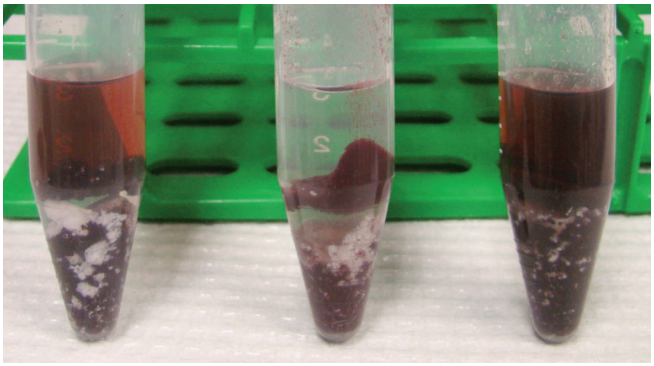
Results and Discussion

The experiments showed that the use of ACN (0.4% FA) as the extraction solvent offered a better lysed sample versus the other extraction solvents where the sample became a solid mass (see Figures 1 and 2). The AOAC buffered salts yielded the cleanest extract, visually (Figure 3) and was chosen for use with the d-SPE containing 50 mg PSA, 150 mg $MgSO_4$ for the extraction of the pharmaceuticals in whole blood (Figure 4). It is worth noting that the d-SPE step does in fact offer substantial clean-up for all the extracted samples, especially from the EN and non-buffered salt extracts, which initially showed a significant amount of red blood cells remaining in the extract.



A B

Figure 1. Addition of ACN (A) or ACN (1% AA) (B) to the whole blood, common solvents used in the QuEChERS method.



A B C

Figure 3. After the addition of ACN (0.4% FA), QuEChERS salts, shake and vortex.
A EN method citrate salts,
B AOAC method acetate salts
C non-buffered method chloride salts

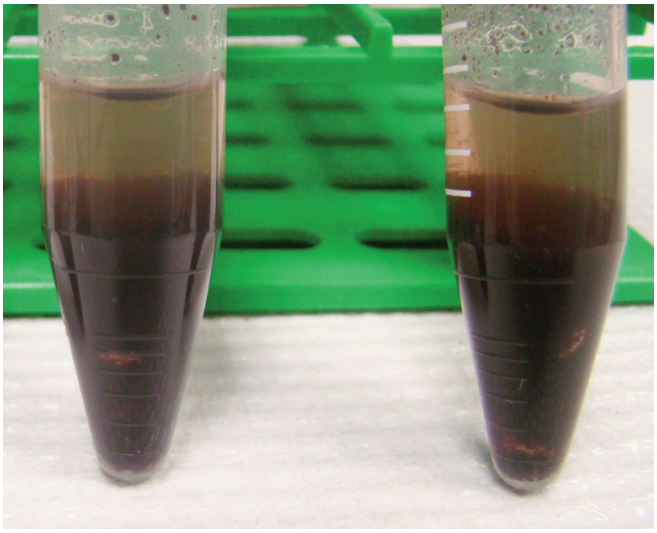
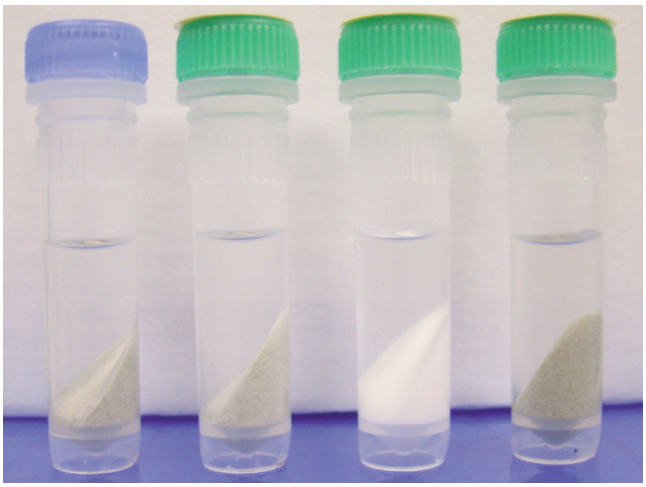


Figure 2. Addition of ACN (0.4% FA) to the whole blood.



A B C D

Figure 4. Extract after the addition of d-SPE clean-up containing 150 mg $MgSO_4$ and varying amounts of PSA.
A EN citrate salts and EN d-SPE 25 mg PSA
B EN citrate salts and AOAC d-SPE 50 mg PSA
C AOAC acetate salts and AOAC d-SPE 50 mg PSA
D Non-buffered chloride salts and AOAC d-SPE 50 mg PSA

The mini-extraction procedure is based on the principles behind the QuEChERS methodology. Its purpose is an alternative to more complicated techniques, offering a "just enough" sample preparation technique for complex matrices such as whole blood. This type of sample preparation technique is extremely complimentary to the powerful selectivity of LC-MS/MS multiple reaction monitoring (MRM) mode. The whole blood extract appeared to be clean and free of impurities, indicating that the blank whole blood extract did not contribute any interferences with target compounds. Figure 5 shows the chromatogram of a 10 ng/mL spiked whole blood sample after the mini-extraction procedure.

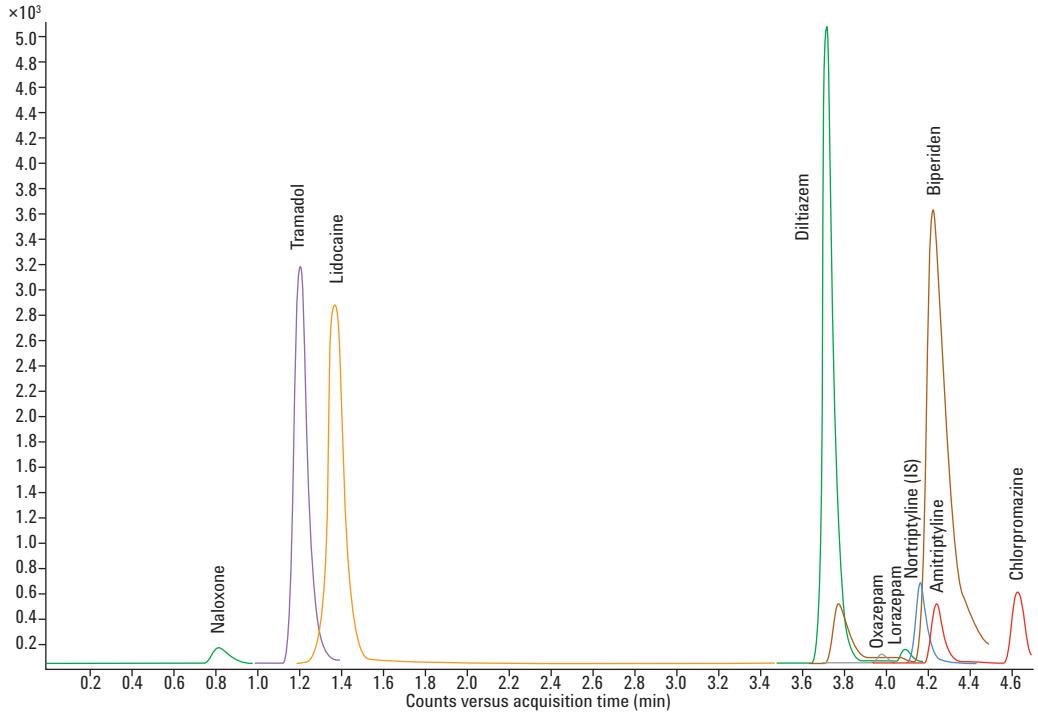


Figure 5. LC-MS/MS chromatograms of 10 ng/mL spiked whole blood sample after mini-extraction; AOAC acetate salts and AOAC d-SPE with 50 mg PSA and 150 mg $MgSO_4$.

Linearity and limit of quantification (LOQ)

The linear calibration range evaluated for all the pharmaceuticals was 10-250 ng/mL. Matrix blank extracts were prepared for the calibration curves. Calibration curves, spiked in the matrix blank extracts, were made at levels of 10, 25, 50, 100, and 250 ng/mL. The Nortriptyline (IS) was used at 100 ng/mL. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte/peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). Figure 6 is an example of the regression equation and correlation coefficient (R^2) observed for the nine pharmaceuticals from whole blood.

Recovery and reproducibility

The recovery and reproducibility were evaluated by spiking standards in the whole blood sample at levels at 25, 50, and 100 ng/mL. These QC samples were quantitated against the matrix spiked calibration curve. The analysis was performed in six replicates at each level. The recovery and reproducibility (RSD) data are shown in Table 4.

It can be seen from the results that all the pharmaceuticals give acceptable recoveries (average > 90%) and precision (average of 7% RSD). We have observed a small degree of matrix interference at low levels of concentration, < 25 ng/mL, with the pharmaceuticals investigated.

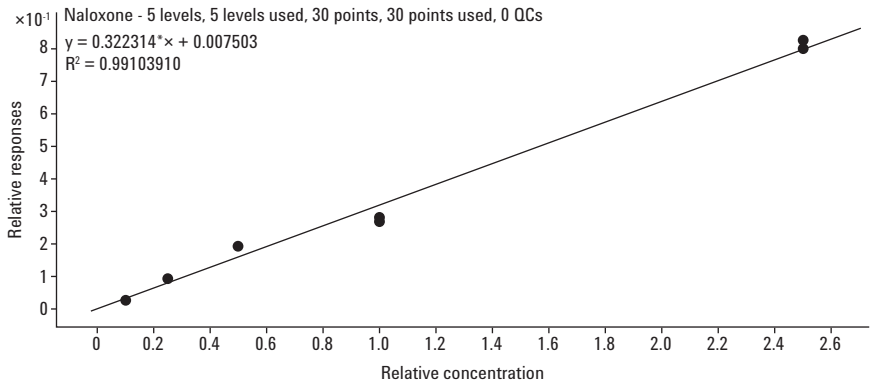


Figure 6. Example of the results from the mini-extraction, standard linear curve for naloxone from 10-250 ng/mL, R^2 = 0.991.

Table 4. Recovery and RSD for the Extracted Drug Compounds

	25 ng/mL Spiked		50 ng/mL Spiked		100 ng/mL Spiked	
Compound	**Recovery**	**RSD**	**Recovery**	**RSD**	**Recovery**	**RSD**
Lidocaine	81.6	35.3	98.7	15.7	100	11.8
Tramadol	97.2	18.6	105	3.0	104	8.2
Amitriptyline	85	13.6	104	2.1	104	8.2
Biperidene	75.5	14.8	97	4.5	99	8.2
Oxazepam	60.4	17.3	77.0	9.2	78	8.6
Lorazepam	68.4	17.0	81.9	6.8	81.8	8.6
Chlorpromazine	75	14.1	110	10.3	105	6.3
Diltiazem	63.7	15.8	88.1	2.7	91.7	8.3
Naloxone	68	12.1	80.6	9.0	75.5	7.7

Conclusion

Mini-extraction sample preparation is a simple, easy, and cost effective approach, requiring minimal sample preparation expertise, solvent, or equipment. The mini-extraction approach for the extraction of pharmaceuticals from whole blood offers an alternative sample preparation technique that can be easily implemented by laboratories. Although matrix interference was observed at low level concentrations for some of the pharmaceuticals, improvements in the method will include a dispersive SPE that contains additional solid phase extraction materials to facilitate matrix removal. The Poroshell 120 EC-C18 column offers different selectivity and exceptional peak shape across the wide range of pharmaceuticals used in this study.

References

1. M. Anastassiades, S.J. Lehotay, D. Štajnbaher and F.J. Schenk, J. (2003) AOAC Int., 86, 4121.
2. G. Stubbings and T. Bigwood (2009) *Anal. Chim. Acta*, 637, 68.
3. R.R. Rasmussen, I.M.L.D. Storm, P.ZH. Rasmussen, J. Smedsgaard and K.F. Nielsen, (2010) *Anal. Bioanal. Chem.*, 397, 765.
4. D. Smith and K. Lynam (2010) GC/µECD analysis and confirmation of PCBs in fish tissue with Agilent J&W DB-35ms and DB-XLB GC columns. Agilent Technologies, Inc., 5990-6236EN.
5. F. Plössl, M. Giera and F. Bracher (2006) *J. Chrom. A*, 1135, 19.

For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.

www.agilent.com/chem

Printed in the USA
October 4, 2011
5990-8789EN

Agilent Technologies