Sensitivity enhancement for potential genotoxic impurity determination using the Agilent 1290 Infinity LC System and a 60 mm Agilent Max-Light Cartridge Cell

Application Note

Drug Development, Drug Discovery

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Abstract

The Agilent 1290 Infinity LC system was used to analyze traces of potential genotoxic impurities (PGIs) in pharmaceuticals. A generic method was developed to separate a set of 10 arylamine and aminopyridine impurities. The Agilent 1290 Infinity Diode-Array Detector (DAD) with a 60 mm Agilent Max-Light Cartridge High Sensitivity Cell was used to obtain maximum sensitivity. Two generic methods were developed to separate a set of 10 target compounds and the performance of these methods was evaluated using standard solutions. Detection limits were as low as 0.2 ng/mL (4 pg on-column). The data were compared with data obtained with a Max-Light Cartridge Standard Cell. Real samples were analyzed with rapid methods, developed for a relevant combination of PGI and active pharmaceutical ingredient (API). Analysis times were approximately 5 minutes. The performance was evaluated with spiked and unspiked samples after a simple sample preparation procedure.

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Introduction

Chromatographic purity analyses are a crucial part of drug development and quality control in pharmaceutical laboratories. Potential genotoxic impurities (PGIs) are a specific group of pharmaceutical impurities, which have recently been subject to increased attention. These impurities are residues from the synthesis process of the drug substance, from the production of the drug formulation, and/or can result from degradation of the active pharmaceutical ingredient (API) or excipiens. Due to their structure and reactivity, PGIs can possess genotoxic activity and lists with structural alert functionalities are available.

Guidelines have been issued by both European and United States drug agencies describing the approach to characterize and reduce the presence of PGIs in drug substances and formulations [1,2]. A threshold of toxicological concern (TTC) value of 1.5 µg/day intake of a genotoxic impurity is considered to be associated with an acceptable risk, that is, the risk of significant genotoxicity is very low[3]. The Committee for Medicinal Products (CHMP) defines an acceptable risk as an additional risk of <1 in 100,000 based on a lifetime exposure to the genotoxic impurity. Taking into account the total drug substance intake, analytical methods should allow the detection of these impurities at low ppm level relative to the API (for example, a 1 ppm PGI level corresponds to a daily intake of 0.5 µg when 500 mg drug substance is administered per day).

Since PGIs cover a wide range of polarities and volatilities, different analytical methods are used. The required detection limits for PGIs are generally significantly lower compared to classical impurity testing. Mass spectrometric detectors are often put on in order to achieve low detection limits demanded in real samples. The use of mass spectrometers in HPLC and UHPLC has several advantages, amongst which the gain in sensitivity and increased selectivity are the most important. A review of methods for various classes of PGIs was recently published together with a method selection chart[4].

A typical class of PGIs is the arylamines and aminopyridines which are frequently used in the synthesis of APIs. The structures of the substances discussed in this application note are shown in Figure 1. Recently, methods were described for these compounds using LC-MS[5] and LC-MS/MS[6]. These systems offer the required sensitivity to detect low traces of PGIs. The influence from the API matrix is minimal due to their outstanding selectivity and specificity. There are however, some significant drawbacks when using these state-of-the-art detectors such as the high purchase investment and costs of operation, the need for skilled and trained staff, and a potential lack of robustness. For these reasons, QC routine labs prefer to use less expensive and complicated techniques such as UV and diode-array detectors. Unfortunately, these types of detectors often have a significantly lower sensitivity compared to modern (for example, triple quadrupole) MS systems.

This Application Note describes the determination of arylamines and aminopyridines by UHPLC in combination with DAD detection. In order to increase the sensitivity of the Agilent 1290 Infinity diode-array detector, the Max-Light Cartridge High Sensitivity Cell was developed. This cell has an optical path length of 60 mm with an internal volume of only 4 µL. Compared to the standard flow cell with its 10 mm path length the sensitivity should, in theory, increase with a factor of 6 (Beer's law).

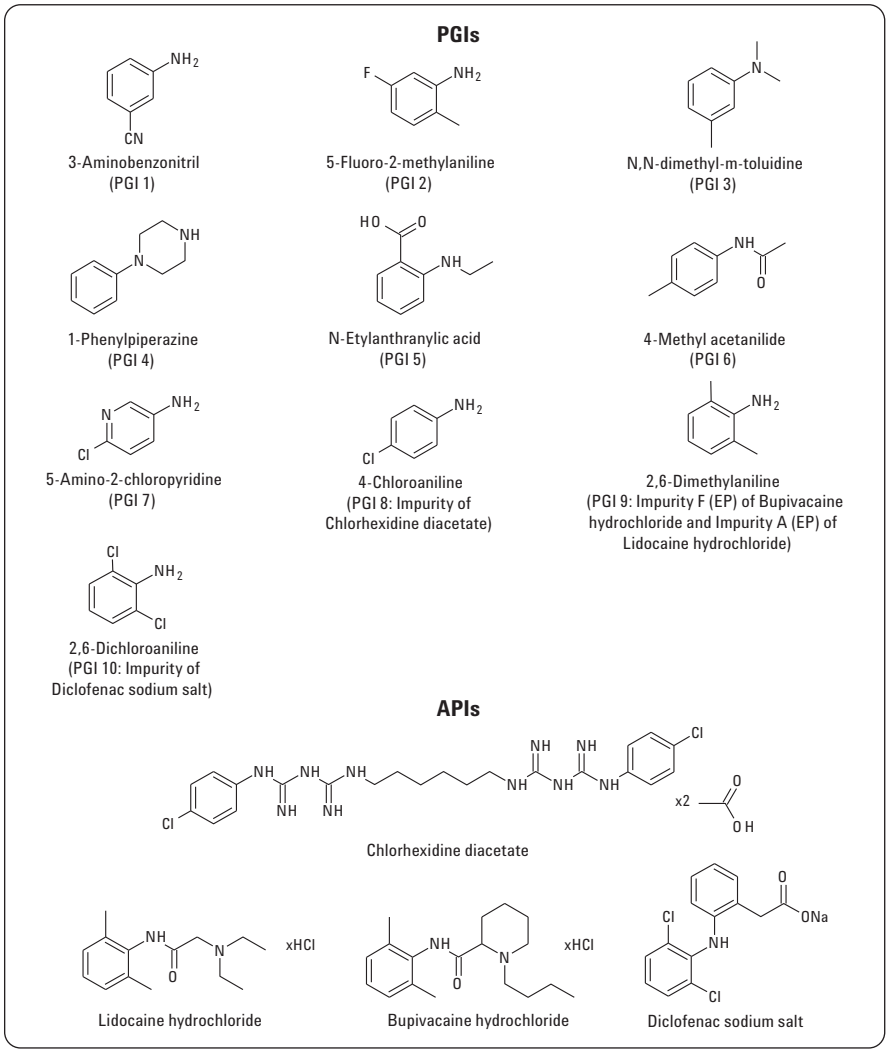

Figure 1
Structures of PGIs and APIs under investigation.

In combination with Agilent ZORBAX RRHD UHPLC columns, providing the necessary peak efficiency and selectivity to avoid matrix interferences of the APIs, detection limits for pharmaceutical trace analysis of PGIs in pharmaceuticals could be drastically decreased without the use of MS.

Experimental

Standards Solutions

A 100 µg/mL mixture of the PGIs was prepared in acetonitrile. This stock solution was stored at –18 °C. Further dilutions in 10% acetonitrile in water were used for the evaluation of the generic method.

Individual solution of PGIs 8, 9, and 10 were prepared in acetonitrile. These solutions were further diluted in acetonitrile to prepare the standard and spiking solutions used for dedicated methods.

Sample Preparation

The following APIs were selected:

- Bupivacaine hydrochloride (Purity min 99%)
- Lidocaine hydrochloride (Purity min 99%)
- Chlorhexidine diacetate (Purity min 97.5%)
- Diclofenac sodium salt (Purity min 98%)

The sample preparation procedure is described below. Some APIs were not completely dissolved after ultrasonic treatment. The solubility of the PGI in the extraction solvent is excellent and spiking experiments have demonstrated that the recovery is higher than 70%[5].

1. Weigh 120 mg sample into a 1.5 mL Eppendorf tube
2. Add spiking solution if necessary
3. Add 1.2 mL acetonitrile (API is at 10%)
4. Vortex, 30 s
5. Ultrasonic batch, 5 min
6. Vortex, 30 s
7. Centrifuge at 13,000 rpm, 2 min
8. Filter solution through a syringe filter (0.2 µm pore size, regenerated cellulose, Agilent p/n 5061-3366)

Equipment

An Agilent 1290 Infinity UHPLC system with the following configuration was used.

Agilent part number	Description
G4220A	Agilent 1290 Infinity Binary Pump with integrated vacuum degasser
G4226A	Agilent 1290 Infinity Autosampler
G1330B	Agilent 1290 Infinity Thermostat
G1316C	Agilent 1290 Infinity Thermostatted Column Compartment
G4212A	Agilent 1290 Infinity Diode Array Detector
G4212-60007	Agilent Max-Light Cartridge High Sensitivity Cell (60 mm optical path length)
G4212-60008	Agilent Max-Light Cartridge Standard Cell (10 mm optical path length)

Chromatographic Conditions

Generic arylamine and aminopyridine method

	Methanol method	Acetonitrile method
Column:	Agilent Eclipse Plus C18 RRHD, 150 mm L × 3.0 mm id, 1.8 µm dp (p/n 959759-302)	
Injection:	20 µL, with needle wash (flushport, 5 s, water/methanol 1/1)	
Sample temperature:	15 °C	
Flow rate:	1 mL/min	
Mobile phase:	A = 5 mM H_3PO_4/NaH_2PO_4 in water, pH 2.75 B = methanol	A = 5 mM H_3PO_4/NaH_2PO_4 in water, pH 2.75 B=acetonitrile
Gradient:	0–0.5 min 10% B 0.5–6.3 min 10–85% B 6.3–6.5 min 85–100% B 6.5–7.5 min 100% B 7.5–9 min 10% B	0–0.5 min 5% B 0.5–6.3 min 5–80% B 6.3–6.5 min 80–100%B 6.5–7.5 min 100% B 7.5–9 min 5% B
Column temperature:	40 °C	35 °C
DAD peak width:	>0.025 min	
DAD signals:	A = Sig 225/5 nm, Ref 450/40 nm (PGI 1, 5) B = Sig 232/5 nm, Ref 450/40 nm (PGI 9, 2) C = Sig 240/10 nm, Ref 450/40 nm (PGI 4, 7, 8, 6) D = Sig 260/10 nm, Ref 450/40 nm (PGI 3) E = Sig 296/10 nm, Ref 450/40 nm (PGI 10)	

Dedicated method (for specific samples)

Column:	Agilent Eclipse Plus C18 RRHD, 100 mm L × 3.0 mm id, 1.8 µm dp (p/n 959758-302)
Mobile phase:	A = 5 mM H_3PO_4/NaH_2PO_4 in water, pH 2.75 B = methanol or acetonitrile
Flow rate:	1.25 mL/min
Column temperature:	40 °C
Injection:	5 µL, with needle wash (flushport, 5 s, water/methanol 1/1)
Sample temperature:	15 °C

		PGI 8	PGI 9	PGI 9	PGI 10	PGI 10
Modifier:		Methanol	Methanol	Acetonitrile	Methanol	Acetonitrile
Gradient:	0–3.5 min	30–100%	25–100%	15–100%	50–100%	35–100%
	3.5–4 min	100%	100%	100%	100%	100%
	4–4.8 min	30%	25%	15	50%	35%
DAD:	Peak width	>0.013 min	>0.013 min	>0.013 min	>0.013 min	>0.013 min
	Signal	240/10 nm	232/5 nm	232/5 nm	296/10 nm	296/10 nm
	Reference	450/40 nm	450/40 nm	450/40 nm	450/40 nm	450/40 nm

Results and discussion

Generic Method

Previously, chromatographic methods were developed for the analysis of arylamines and aminopyridines using LC-MS[5] and LC-MS/MS[6]. In those methods, volatile mobile phase components were used. In order to optimize the mobile phase for highly sensitive diode-array detection, these methods were translated. The UV absorbing formic acid was replaced by a UV transparent phosphate buffer at the same pH and with a similar ionic strength. The chromatographic selectivity after this adjustment was very similar to the original LC-MS method. Additionally, the column internal diameter, and consequently, the flow rate were increased to maintain the high efficiency when a larger detection cell volume is employed.

When using MS detection, chromatographic separation of target PGIs is less critical for their determination, since target compounds can be measured if the respective ions or ion transitions are different. Since this detector selectivity is no longer present with DAD, chromatographic resolution is needed. Two generic methods were developed for the complete separation of the 10 selected PGIs. The methods differ in the organic mobile phase component (methanol or acetonitrile) and gradient composition. Changing the organic modifier has significant effect on the selectivity and can be applied to resolve target solutes from each other and from the matrix (drug substance)[5].

An example of the analysis of a standard solution with the high sensitivity and the standard flow cell is shown in Figure 2, respectively using acetonitrile and methanol as organic mobile phase. The increased sensitivity is obvious by comparing the chromatograms obtained by the high sensitivity cell with the standard cell. This improvement in sensitivity, however, also affects the baseline. All baseline fluctuations and drift caused by impurities in the sample, system or mobile phases are in the same way enlarged as the target compounds. This is illustrated by the impurity at 3.6 min in the acetonitrile chromatogram, which is visible in the upper trace and not in the trace obtained by the standard cell. In the same way, an impurity is detected at 5 min using methanol and the high sensitivity cell. Both impurities were also detected in blank runs and are thus not sample related. Care has to be taken that all solvents are as clean as possible. The high sensitivity flow cell clearly puts higher demands on solvent purity and, in certain cases, measures need to be taken to enhance the chromatographic selectivity for a given analysis.

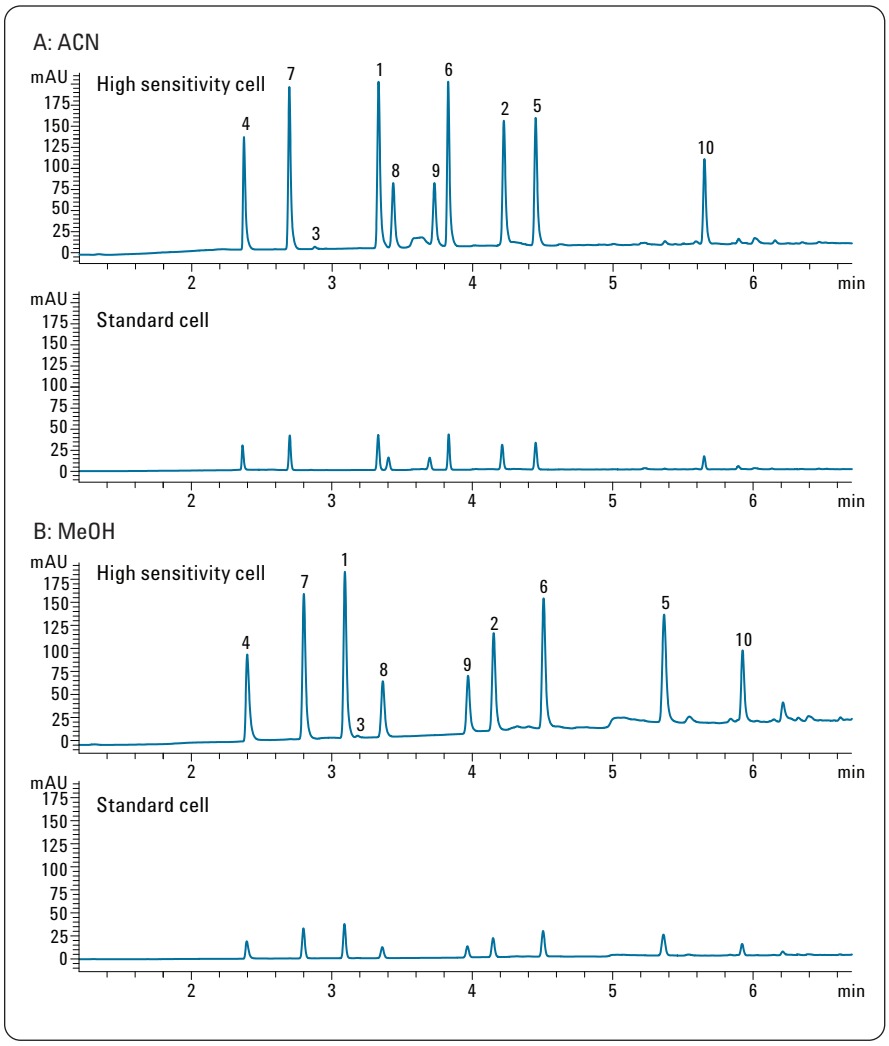

Figure 2
Comparison of the 10 mm standard DAD cell and the 60 mm high sensitivity DAD cell for a 0.5 µg/mL standard mixture of all PGIs analyzed on the 150 mm column with the generic method. DAD wavelength: 232 nm. A: acetonitrile mobile phase B, B. methanol mobile phase B.

The performance of the methods was evaluated with standard solutions of the selected PGIs. Linearity was measured by single injections of different concentration levels and repeatability of injection was calculated from five consecutive injections at various levels (0.01, 0.1, and 1 µg/mL). A summary of the method validation data is given in Table 1. The detection limits were determined with both a high sensitivity and a standard DAD flow cell. The influence of the 6-fold longer optic path length is obvious. The detection limits are significantly lower. For some impurities, the sensitivity increase is typically a factor of 5 or in some cases even higher (for example, PGI 10). Interestingly, the observed detection limits for several target PGI with the 60 mm flow cell are in the same order as previously determined detection limits with Agilent 6400 Series Triple Quadrupole LC/MS system[6].

For some compounds however the detection limits are considerably higher than for others, This is mostly caused by baseline disturbances and system peaks which interfere with the detection of trace levels of these PGIs (marked with * in Table 1). For detection of these PGIs, the method should be optimized individually, resulting in lower detection limits.

An example of an analysis of a standard solution at 0.1 ppm (0.1 µg/g API = 0.01 µg/mL in solution) concentration level is shown in Figure 3. This chromatogram shows the signal at the optimal detection wavelength for each PGI selected.

	Repeatability (%RSD)			Linearity		LOD (ng/mL)	
Acetonitrile	**0.01 µg/mL**	**0.1 µg/mL**	**1 µg/mL**	**Range**	**R^2**	**60 mm**	**10 mm**
PGI 1	0.48	0.19	0.05	0.5–200	1.0000	0.2	0.5
PGI 2	2.38	0.50	0.04	2–200	0.9959	2*	5
PGI 3	3.22	1.79	1.51	5–200	0.9997	5	20
PGI 4	0.58	0.25	0.12	0.5–200	1.0000	0.2	1
PGI 5	0.35	0.25	0.09	0.5–200	1.0000	0.2	1
PGI 6	0.61	0.14	0.02	0.5–200	0.9999	0.2	0.5
PGI 7	0.66	0.07	0.01	0.5–200	1.0000	0.2	0.5
PGI 8	0.63	0.42	0.12	0.5–200	1.0000	0.5	2
PGI 9	8.96	2.34	0.15	5–200	0.9996	2*	5
PGI 10	1.66	0.19	0.15	0.5–200	1.0000	0.2	2
	Repeatability (%RSD)			**Linearity**		**LOD (ng/mL)**	
Methanol	**0.01 µg/mL**	**0.1 µg/mL**	**1 µg/mL**	**Range**	**R^2**	**60 mm**	**10 mm**
PGI 1	2.11	0.07	0.06	0.5–200	0.9997	0.2	0.5
PGI 2	13.26	4.03	0.12	1–200	0.9994	0.5	2
PGI 3	– (=LOD)	2.89	0.75	10–200	0.9985	10	20
PGI 4	4.79	0.44	0.09	1–200	0.9999	0.5	2
PGI 5	1.73	0.89	0.36	0.5–200	0.9994	0.2	1
PGI 6	4.58	2.23	0.06	5–200	0.9997	5*	10
PGI 7	1.46	0.55	0.03	0.5–200	0.9999	0.2	0.5
PGI 8	1.67	0.24	0.22	0.5–200	1.0000	0.2	1
PGI 9	13.87	4.45	0.21	2–200	0.9997	2*	5
PGI 10	2.51	0.42	0.23	1–200	0.9992	1	5

*LOD high due ot interference on baseline.

Table 1
Performance of the Generic Method.

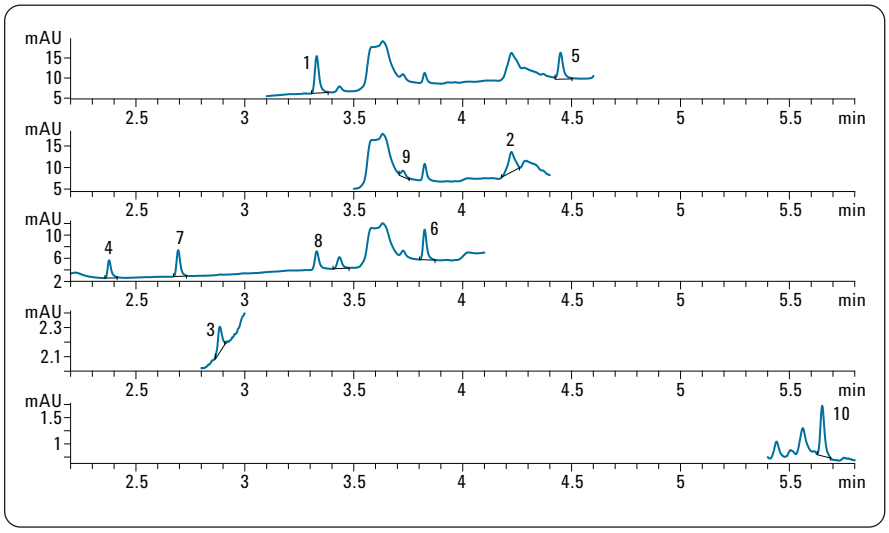

Figure 3
Analysis of a 0.01 µg/mL standard mixture of all PGIs with the 60 mm high sensitivity DAD cell on the 150 mm column with the generic method with acetonitrile as organic modifier.

Dedicated Methods

For the determination of PGI in real samples, possible interference from the active pharmaceutical ingredient (or more abundant other impurities) should be avoided. In order to demonstrate the applicability of the Agilent 1290 Infinity LC setup for real samples, a selection of APIs was analyzed for the determination of their respective known PGIs. After an initial screening with the generic methods, dedicated methods using a specific mobile phase gradient and detector wavelength were developed for each set of API/PGI. For all dedicated methods a shorter (10 cm) column was used, since not all PGIs have to be separated from each other and analysis time is one of the key parameters in pharmaceutical quality control. The dedicated methods, (see experimental) have cycle times of ca. 5 min which is definitely acceptable for high productivity analyses.

From 4 selected pharmaceuticals, non-spiked and spiked solutions were analyzed. Spiking levels were from 0.1 to 100 ppm (µg/g API). The measured concentration in the spiked samples was compared to the theoretical (spiked) concentration to determine the recovery. Some API samples already contained significant amounts of PGI. As a consequence, the PGI recovery cannot be calculated for these samples at low levels.

The measured concentrations and recoveries for PGI 8 in chlorhexidine, PGI 9 in lidocaine and bupivacaine, and PGI 10 in diclofenac are given in Table 2. In some cases, both the results obtained using acetonitrile as mobile phase B and using methanol as mobile phase B are given. Most values are close to 100% and the values for the 0.1 ppm spike are between 80% and 134% which is satisfactory at this level. PGI 10 could be detected at levels as low as 0.01 µg/mL in diclofenac (0.1 ppm relative to API). Some representative chromatograms of samples and standards are given in Figure 4.

	Unspiked	Spike 0.1 ppm	Spike 1 ppm	Spike 10 ppm	Spike 100 ppm
PGI 8 in Chlorhexidine, Methanol, 240 nm					
Concentration (ppm)	42.98	Not	Not	54.71	143.97
Recovery %		relevant	relevant	117.3	101.0
PGI 9 in Lidocaine, Methanol, 232 nm					
Concentration (ppm)	3.46	Not	4.38	12.12	96.08
Recovery %		relevant	91.4	86.5	92.6
PGI 9 in Lidocaine, Acetonitrile, 232 nm					
Concentration (ppm)	4.54	Not	5.82	12.98	95.09
Recovery %		relevant	128.2	84.4	90.5
PGI 9 in Bupivacaine, Acetonitrile, 232 nm					
Concentration (ppm)	0.00	Not	1.05	9.58	99.07
Recovery %		detected	104.5	95.8	99.1
PGI 10 in Diclofenac, Methanol, 296 nm					
Concentration (ppm)	0.00	0.13	1.07	10.26	101.40
Recovery %		134.0	107.2	102.6	101.4
PGI 10 in Diclofenac, Acetonitrile, 296 nm					
Concentration (ppm)	0.07	0.15	1.05	10.33	100.49
Recovery %		80.0	97.9	102.6	100.4

Table 2
Performance of the dedicated methods for the analysis of APIs as such and after spiking. (ppm is concentration relative to API. API is at 10% in sample solution).

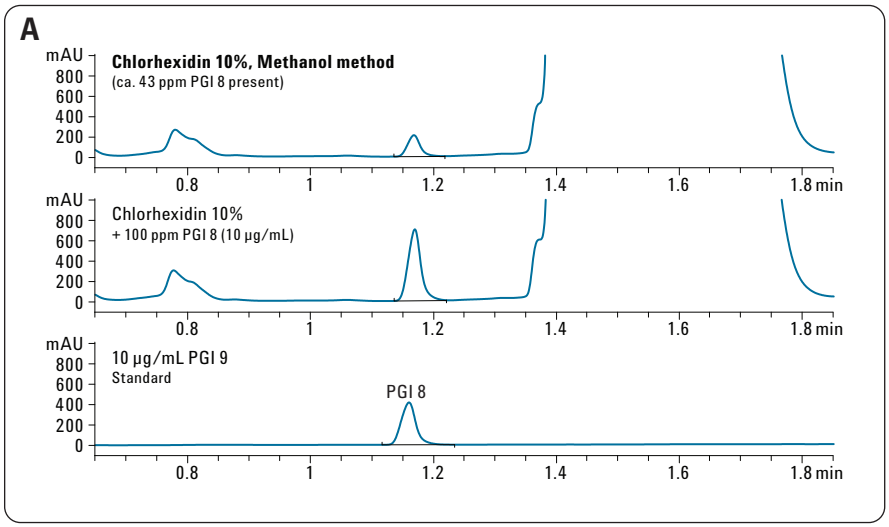

Figure 4
Representative chromatograms of samples, spiked samples, and standard solutions analyzed with dedicated methods. A: PGI 8 in chlorhexidine B: PGI 9 in bupivacain C: PGI 10 in diclofenac. (Continued)

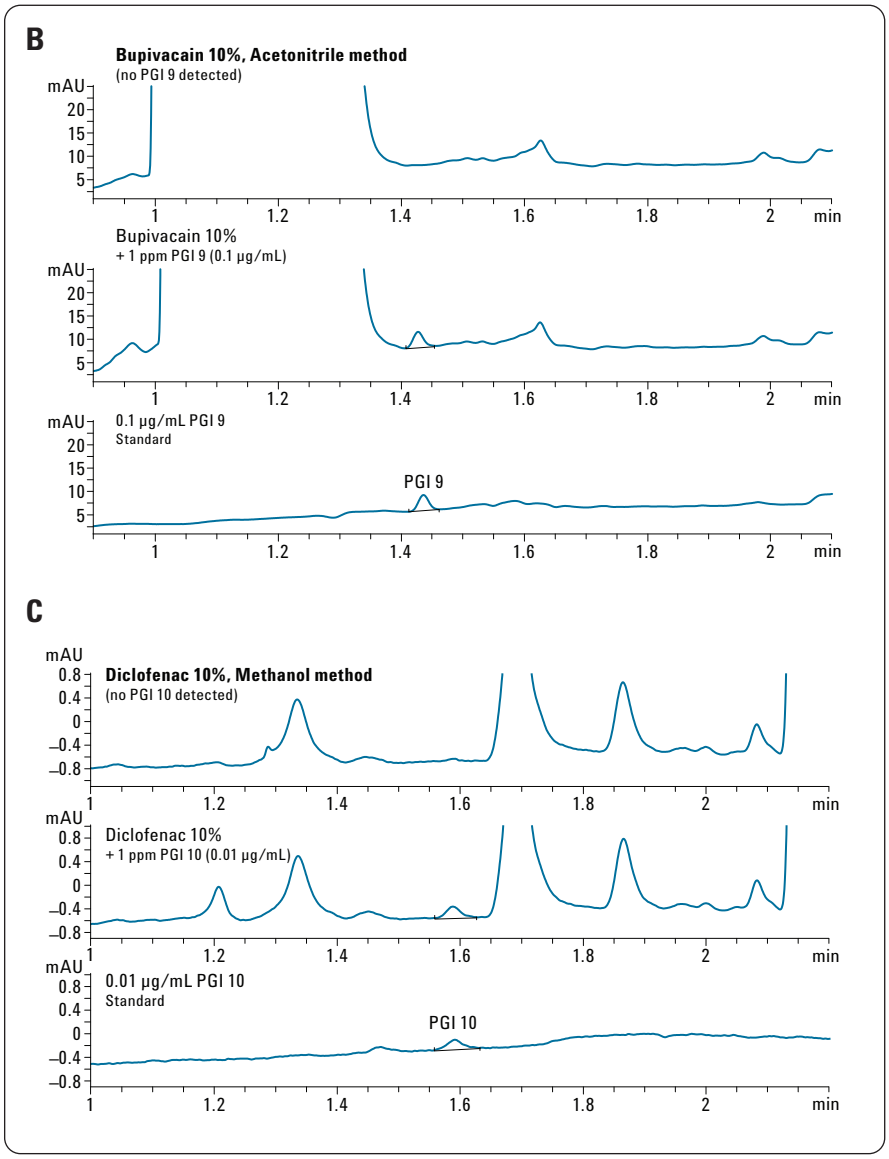

Figure 4
Representative chromatograms of samples, spiked samples, and standard solutions analyzed with dedicated methods. A: PGI 8 in chlorhexidine B: PGI 9 in bupivacain C: PGI 10 in diclofenac.

Conclusion

This Application Note clearly indicates that the Agilent 1290 Infinity LC-DAD in combination with the 60 mm Agilent Max-Light Cartridge High Sensitivity Cell is a valuable tool for routine trace analysis of PGIs in APIs. The use of a non-MS based system for this type of analysis is an attractive alternative for expensive and more complex LC-MS and LC-MS/MS systems.

With a generic method, impurities could be detected at levels as low as 4 pg injected on-column in standard solutions. Dedicated methods were developed for a set of APIs enabling the fast analysis of PGIs in real samples at relevant levels.

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March 1, 2011
Publication Number 5990-7443EN

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