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Comprehensive Analysis of Drugs of Abuse in Blood and Urine with Automated Disposable Pipette Extraction and HPLC/MS/MS

Sparkle T. Ellison, William E. Brewer, Stephen L. Morgan
Department of Chemistry and Biochemistry, University of South Carolina, 631 Sumter Street, Columbia, SC 29208, USA

Fred D. Foster
Gerstel, Inc., 701 Digital Dr. Suite J, Linthicum, MD 21090, USA

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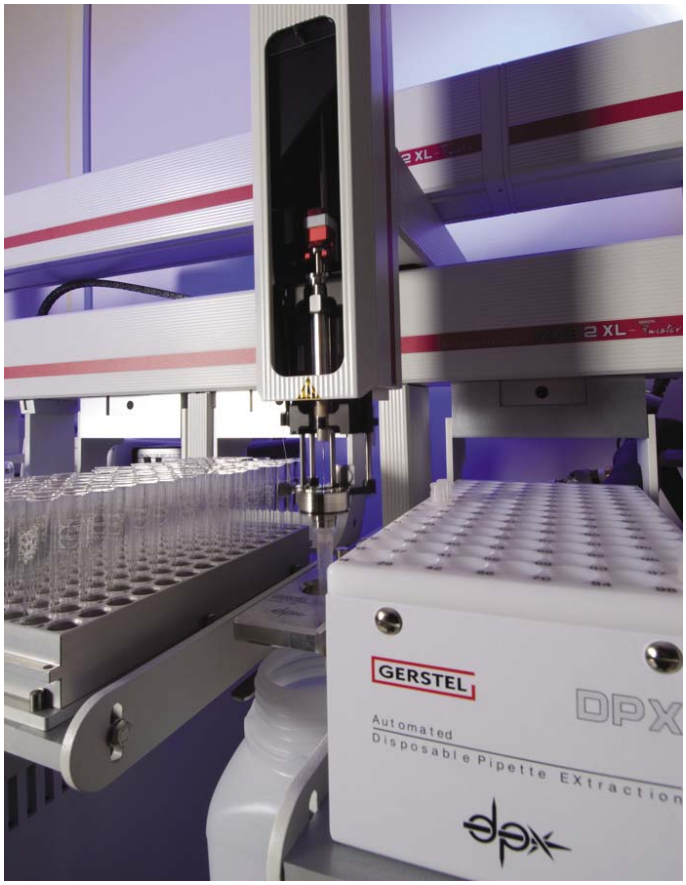
Abstract
The analysis of opiates in blood and urine was accomplished using automated Disposable Pipette Extraction (DPX) followed by LC/MS analysis. The automated extractions were performed in about 5 minutes using a GERSTEL MultiPurpose Sampler (MPS). The eluents were subsequently dried and reconstituted in solvent and injected into a HPLC/MS/MS instrument. Recoveries ranged from 60 to 85% for the opiates in blood, and recoveries ranged from 78 to 85% for the opiates in hydrolyzed urine. The %RSDs were lower than 6% for all analytes.

Introduction
In order to analyze biological specimens for drugs and their metabolites, it is necessary to perform sample preparation to eliminate matrix interference. Solid-phase extraction is generally the preferred sample preparation technique, in this study Disposable Pipette Extraction (DPX) was utilized. DPX is a novel dispersive solid-phase extraction technique that uses loosely contained sorbent in a disposable pipette tip. The sample is aspirated into the tip where it is actively mixed

with the sorbent and forms a suspension. The main advantages of the DPX technology are that the extraction is very rapid, minimal solvent waste is generated, and the entire process can be fully automated including introduction of the extract to the chromatographic system. The GERSTEL MPS autosampler performs DPX extractions in approximately 5 minutes using reversed phase (DPX-RP) or cation exchange (DPX-CX) sorbent material.

For chemical analysis of target drugs, GC/MS or HPLC/MS/MS are generally the preferred techniques. The advantage of LC/MS/MS is that chemical derivatization of the analytes is not required, making sample preparation simpler and less time consuming. In addition, highly efficient ionization, in combination with tandem mass spectrometry results in high sensitivity and selectivity. This study focused on performing automated extraction of reduced sample volumes coupled with LC/MS/MS to provide high throughput analysis "one sample at a time". The sample preparation time was decreased sufficiently to allow the extraction of a sample during the chromatographic analysis of the previous sample in the sequence.



GERSTEL MPS dual rail PrepStation with DPX option

EXPERIMENTAL

Instrumentation. Sample extraction and introduction was automated using a GERSTEL MPS dual rail PrepStation with DPX option (GERSTEL, Linthicum, MD). The HPLC instrument utilized was an Acquity UPLC (Waters, Milford, MA). The MS system used was a Waters Micromass Quattro Premier XE.

Analysis conditions LC.

Mobile Phase:	A - 4.5 mM Ammonium acetate	
	B - Methanol	
Gradient:	Initial	90 % A / 10 % B
	2 min	85 % A / 15 % B
	3 min	65 % A / 35 % B
	4 min	50 % A / 50 % B
	6 min	35 % A / 65 % B
	8 min	90 % A / 10 % B
	(4 min)	
Flowrate:	350 µL/min	
Column:	2.1 mm x 30 mm, 3.5 mm, Eclipse XDB C18 (Agilent)	
Inj. volume:	10 µL	

Analysis conditions MS.

Positive ion mode, Single reaction monitoring

Run time:	10 min
Capillary:	3 kV
Extractor:	2.81 V
Source Temp.:	130 °C
Desolvation Temp.:	391 °C

Compound	M + H [m/z]	Dwell Time [ms]	Cone Voltage [V]
d_3-Oxymorphone	305	50	30
Oxymorphone	302	50	30
Morphine	286	50	30
Hydromorphone	286	50	30
d_3-Oxycodone	319	50	30
Oxycodone	316	50	30
6-MAM	328	50	30
Codeine	300	50	30
Hydrocodone	300	50	30

Sample preparation. All opiate standards were obtained from Cerilliant (Round Rock, TX). A 10 ppm stock solution was prepared in methanol for all sample fortifications. Two internal standards were used, d_3-Oxymorphone for quantitation of Oxymorphone and d_3-Oxycodone for all other opiates. All solvents used were of HPLC grade or higher.

Blood sample preparation. A 250 µL blood sample was spiked at the specified concentration with the stock opiates mix. To precipitate proteins, 500 µL of acetonitrile was added and the solution was vortex mixed and centrifuged. The supernatant was decanted into a clean labeled sample tube. 100 µL of 0.1 M HCl was added to the solution, and the sample tube was placed on the MPS 2 sample tray for automated DPX extraction.

Urine sample preparation. 50 µL of 0.6 M sodium acetate buffer (pH = 4) and 10 µL of ß-glucuronidase was added to a 200 µL sample of urine. The solution was thermostated at 70°C for 2 hours, and then cooled to room temperature. To precipitate proteins, 250 µL of acetonitrile was added to the hydrolyzed urine and the sample was vortex mixed and centrifuged. The supernatant was decanted into a clean labeled sample tube. 200 µL of 0.1 M HCl was added to the sample solution, and the sample tube was placed on the MPS sample tray for automated DPX extraction.

Extraction. A GERSTEL MPS dual rail PrepStation was set up with 1 mL DPX-CX tips (DPX Labs, LLC, Columbia, SC) for extraction of drugs from blood and hydrolyzed urine. The following automation method was used: 250 µL of 30% acetonitrile/water was slowly added through the top of the DPX tip at a rate of 50 µL/s to wet the sorbent. The sample was then aspirated into the DPX tip at a rate of 90 µL/s and mixed with the sorbent by drawing in an additional 2 mL of air. After a 30 s equilibration time to allow analyte binding, the resulting solution was dispensed to waste. To wash off excess matrix, a 500 µL wash of 10% acetonitrile/water was added to the sorbent material through the top of the DPX tip and dispensed to waste followed by an additional wash using 500 µL of acetone. For elution of the analytes, 700 µL of 78/20/2 (v/v) of methylene chloride/isopropanol/ammonium hydroxide was added to the sorbent material through the top of the DPX tip and dispensed directly into a clean HPLC vial.

All eluents were dried and reconstituted with 100 µL of methanol and 400 µL of 4.5mM ammonium acetate before injection.

Results and Discussion

The DPX-CX extractions were readily performed using the GERSTEL MPS dual rail PrepStation. These DPX tips are ideal for basic drugs due to their mixed-mode cation exchange and reversed phase characteristics. The entire extraction process took approximately 5 minutes per sample. Because a basic eluent is used with the cation exchange sorbent, the eluents had to be solvent exchanged into the HPLC mobile phase. The extract was dried in about 4 minutes using low heat and nitrogen gas flow.

All HPLC/MS spectra were collected using single reaction monitoring (SRM) because under the HPLC conditions used we were unable to generate quality daughter ions for the opiate drugs using multiple reaction monitoring (MRM). Although SRM MS analysis may not provide the best sensitivity for the analysis of these drugs at low concentrations, this study focused on the automated DPX extraction and the utility of this automated sample preparation for HPLC/MS analysis of opiates.

A rapid resolution HPLC column was chosen to generate chromatographic data in less than 10 minutes. Figure 1 shows that the chromatogram of a DPX-extracted whole blood blank is free from any peaks that could interfere with the opiates.

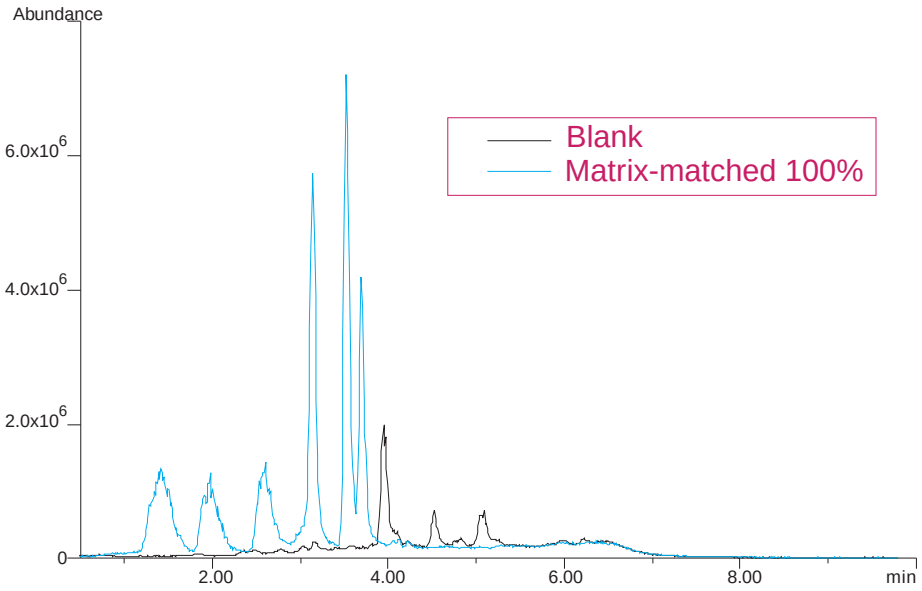


Figure 1. Overlay total ion LC/MS chromatograms of DPX extracts of a blank blood sample and of a matrix-matched sample, both spiked at 0.5 ppm. The overlay shows that interferences are negligible

Figure 2 shows the extracted ion chromatogram of a DPX extract of whole blood spiked with 400 ppb of opiates. The chromatogram is free from interferences, the opiates were extracted reproducibly and with high recoveries (Table 1). It is noteworthy that this blood sample was only 0.25 mL.

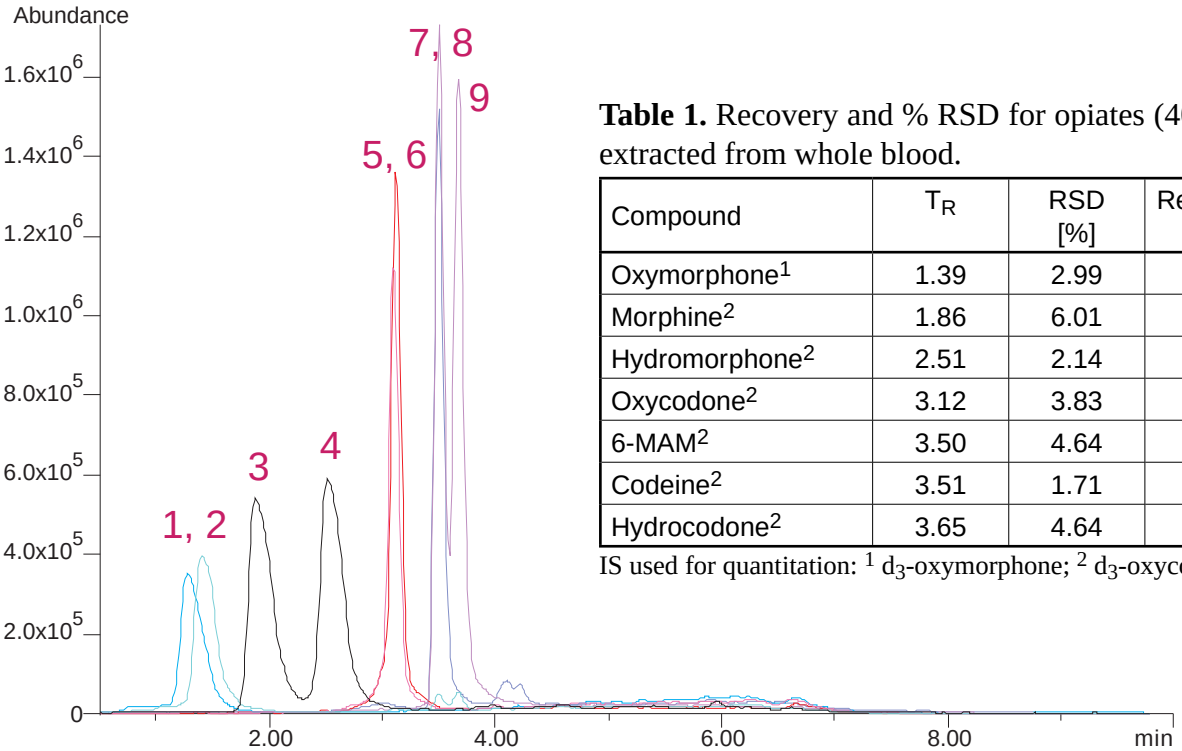


Table 1. Recovery and % RSD for opiates (400 ppb) extracted from whole blood.

Compound	T_R	RSD [%]	Recovery [%]
Oxymorphone[1]	1.39	2.99	57.5
Morphine[2]	1.86	6.01	62.4
Hydromorphone[2]	2.51	2.14	63.7
Oxycodone[2]	3.12	3.83	74.2
6-MAM[2]	3.50	4.64	77.4
Codeine[2]	3.51	1.71	85.1
Hydrocodone[2]	3.65	4.64	70.7

IS used for quantitation: [1] d_3-oxymorphone; [2] d_3-oxycodone

Figure 2. Extracted ion chromatogram of a DPX extract of whole blood spiked with 400 ppb opiate mix. Peak Identities: (1) d_3-oxymorphone, (2) oxymorphone, (3) morphine, (4) hydromorphone, (5) d_3-oxycodone, (6) oxycodone, (7) 6-MAM, (8) codeine, and (9) hydrocodone.

Figure 3 shows a chromatogram of opiates in whole blood at a concentration of 100 ppb. Even when performing SRM MS analysis, the sensitivity is more than sufficient, demonstrating the high ionization efficiency of the electrospray system. Most importantly, no matrix effect or ion suppression was observed, showing that sample extraction and cleanup with DPX is well suited for the analysis.

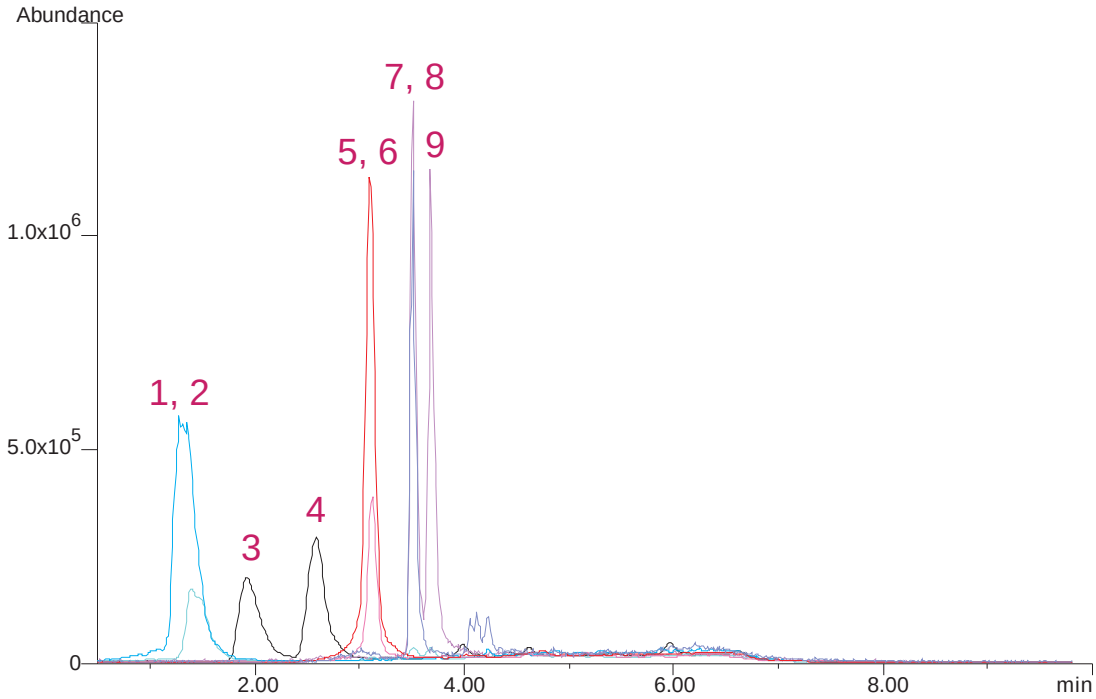


Figure 3. Extracted ion chromatogram of a DPX extract of whole blood spiked with 100 ppb of the opiate mix and with 400 ppb of the internal standards (d_3-oxymorphone and d_3-oxycodone) in whole blood. Peak Identities: (1) d_3-oxymorphone, (2) oxymorphone, (3) morphine, (4) hydromorphone, (5) d_3-oxycodone, (6) oxycodone, (7) 6-MAM, (8) codeine, and (9) hydrocodone.

Figure 4 shows the analysis of urine spiked with 500 ppb of opiates. Again, extracts were free from interferences. No matrix effect or ion suppression for opiates was seen. Table 2 shows that the DPX extractions yielded high recoveries with very good reproducibility.

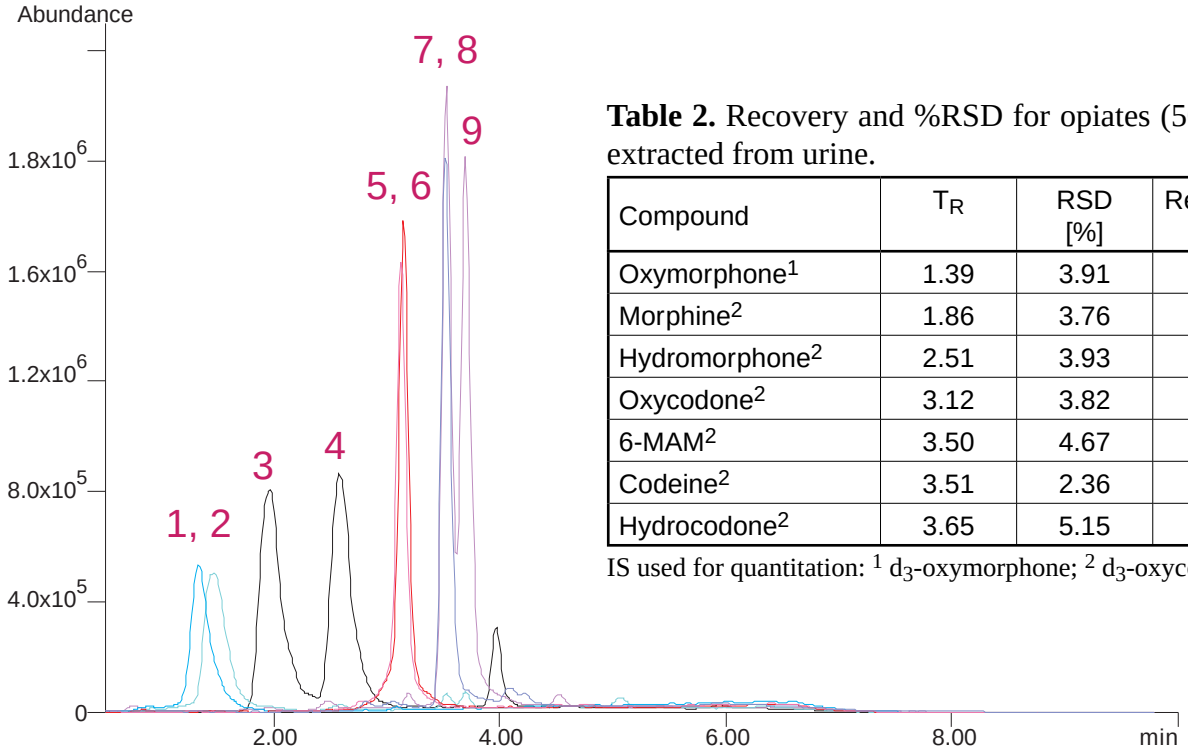


Table 2. Recovery and %RSD for opiates (500 ppb) extracted from urine.

Compound	T_R	RSD [%]	Recovery [%]
Oxymorphone[1]	1.39	3.91	78.3
Morphine[2]	1.86	3.76	81.1
Hydromorphone[2]	2.51	3.93	82.0
Oxycodone[2]	3.12	3.82	80.6
6-MAM[2]	3.50	4.67	81.5
Codeine[2]	3.51	2.36	83.5
Hydrocodone[2]	3.65	5.15	84.8

IS used for quantitation: [1] d_3-oxymorphone; [2] d_3-oxycodone

Figure 4. Extracted ion chromatogram of the DPX extract of 500 ppb of the opiate mix spiked in hydrolyzed urine. Peak Identities: (1) d_3-oxymorphone, (2) oxymorphone, (3) morphine, (4) hydromorphone, (5) d_3-oxycodone, (6) oxycodone, (7) 6-MAM, (8) codeine, and (9) hydrocodone.

Conclusion

Automated DPX extraction of opiates from biological specimens can be performed successfully using the GERSTEL MPS dual rail PrepStation. In the work presented here, the total extraction time was 5 minutes, additionally 4 minutes were required for evaporation and solvent exchange. The total sample preparation time was less than the chromatographic run time, which means that the next sample can be prepared while separation of the current sample is in progress. Whenever the LC/MS/MS system has finished a run, the next sample is ready to be introduced ensuring the highest possible throughput. Additionally, "just in time" sample preparation helps to ensure that the prepared sample is not kept in the autosampler for a long time prior to being analyzed, reducing the risk of analyte degradation and helping to maintain sample integrity.

The DPX-CX tips work very well for extraction of opiates, recoveries were in the range from 60 to 85% with RSD's below 6%. Future work will focus on determining the lower limits of detection and quantitation using tandem mass spectrometry with multiple reaction monitoring. Also, automated DPX combined with HPLC/MS/MS will be optimized for other drugs and metabolites.

GERSTEL GmbH & Co. KG
Eberhard-Gerstel-Platz 1
45473 Mülheim an der Ruhr
Germany
+49 (0) 208 - 7 65 03-0
+49 (0) 208 - 7 65 03 33
gerstel@gerstel.com
www.gerstel.com

GERSTEL Worldwide

GERSTEL, Inc.
701 Digital Drive, Suite J
Linthicum, MD 21090
USA
+1 (410) 247 5885
+1 (410) 247 5887
sales@gerstelus.com
www.gerstelus.com

GERSTEL AG
Wassergrabe 27
CH-6210 Sursee
Switzerland
+41 (41) 9 21 97 23
+41 (41) 9 21 97 25
swiss@ch.gerstel.com
www.gerstel.ch

GERSTEL K.K.
1-3-1 Nakane, Meguro-ku
Tokyo 152-0031
SMBC Toritsudai Ekimae Bldg 4F
Japan
+81 3 5731 5321
+81 3 5731 5322
info@gerstel.co.jp
www.gerstel.co.jp

GERSTEL LLP
10 Science Park Road
#02-18 The Alpha
Singapore 117684
+65 6779 0933
+65 6779 0938
SEA@gerstel.com
www.gerstel.com

GERSTEL (Shanghai) Co. Ltd
Room 206, 2F, Bldg.56
No.1000, Jinhai Road,
Pudong District
Shanghai 201206
+86 21 50 93 30 57
china@gerstel.com
www.gerstel.cn

GERSTEL Brasil
Av. Pascoal da Rocha Falcão, 367
04785-000 São Paulo - SP Brasil
+55 (11)5665-8931
+55 (11)5666-9084
gerstel-brasil@gerstel.com
www.gerstel.com.br

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