The Development of a Yeast-based Paper Analytical Device (Bio PAD) for the Detection of Prednisone and Prednisolone in Nepali Pharmaceutical Samples

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Background

- Effective pharmaceuticals are essential for proper healthcare, but compromised medicines are found in both high and lower income countries[1]
- Inexpensive, rapid, easy to use, and scalable devices are being developed to test for substandard and falsified medical products[2]
- Prednisolone: a glucocorticoid steroid used to treat arthritis/joint pain, COPD, malaise; but also may have side effects like osteoporosis and birth defects.
  - may be inappropriately dosed in allopathic meds (pharmaceuticals) or added to ayurvedic (holistic healing system) meds
- Nepal: area of interest for counterfeit medicines; bordered by known producers of (up to 97%)[3] of counterfeit medicines, China and India
- Screening methods include paper-based analytical devices (PADs), Raman spectroscopy, and liquid chromatography – mass spectrometry (LC-MS)[4]

Objective

Assess the quality of a sample of Nepali medicines utilizing yeast paper-based analytical devices (Bio PADs), Raman Spectrometry, and Liquid Chromatography – Mass Spectrometry (LC-MS) to detect prednisone or prednisolone in allopathic and ayurvedic medicines.
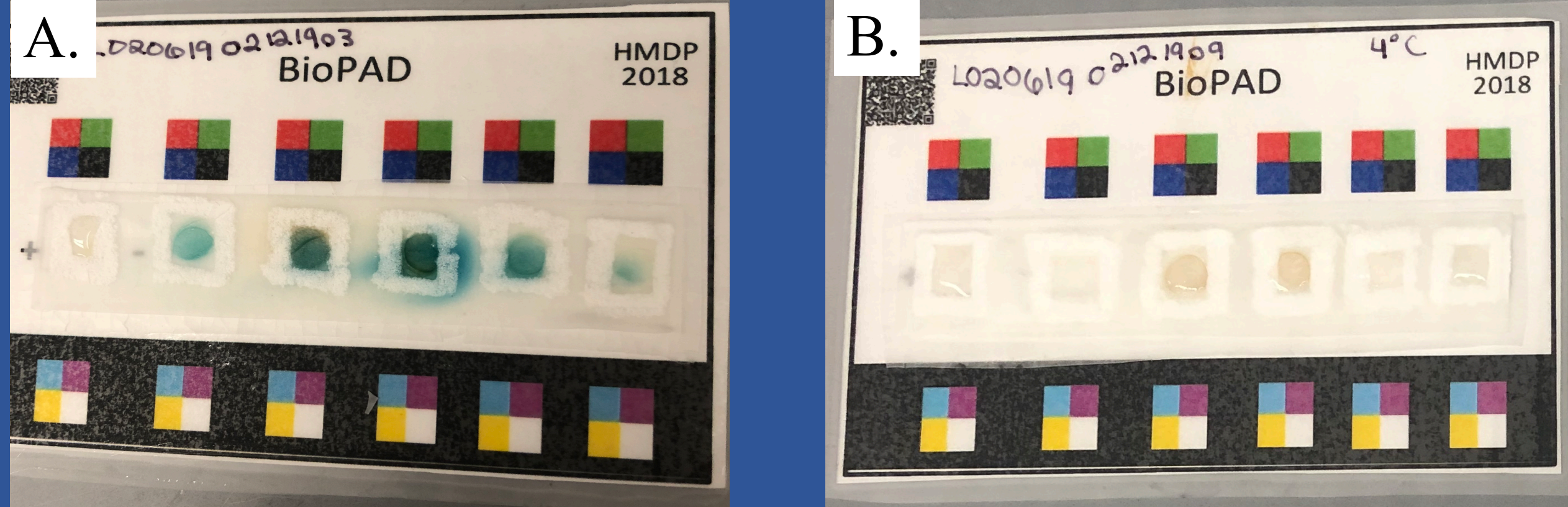
Materials and Methods

- Sampling
  - World Health Organization (WHO) guidelines: 30 samples per region were collected[5]
- Bio PADs
  - Samples extraction: 50% ethanol
  - 8ul (~40μg) applied to yeast
  - 2 yeast strains used for each sample
- Raman Spectroscopy (Mira Cal 3.0)
  - Tablet
  - Crushed samples w/ mortar and pestle
- Liquid Chromatography-Mass Spectrometry
  - Crushed pill in Falcon tube
 - Extracted with LC-grade methanol
 - Filtered into LC vials

Results
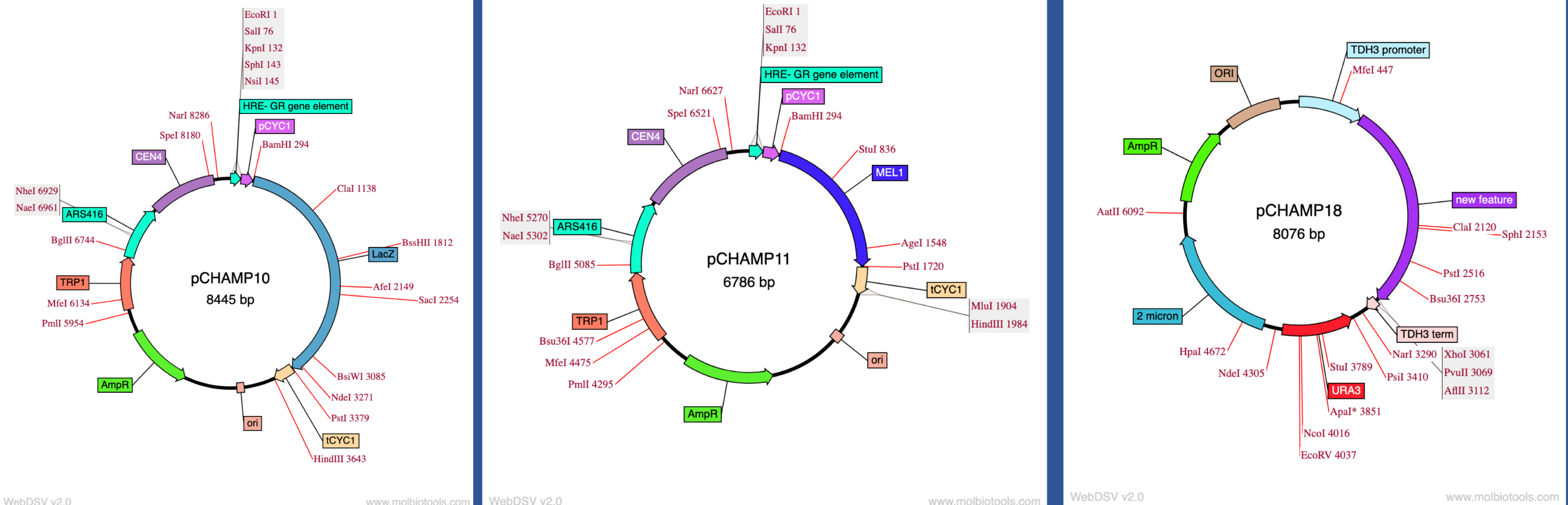
pCHAMP10 and 11 use a glucocorticoid promotor(GRE) to activate *LacZ* or *MEL1* reporter genes, respectively. pCHAMP10 was created by replacing the tetracycline regulated promoter of pCM176[6] with a 134 bp PCR amplified hormone response element (HRE) from plasmid pRR-GR-5Z[7]. pCHAMP11 was created by replacing the *LacZ* reporter gene of pCHAMP10 with a 1416 bp PCR amplified *MEL1* reporter gene from plasmid pCL1[8]. pCHAMP18, which produces the glucocorticoid receptor(GR), was created by inserting the 2334bp PCR amplified GR fragment from pRR-GR-5Z into the pYEpGAP-Cherry plasmid[9], replacing the *mCherry* gene.

Nepal: 152 allopathic & 128 ayurvedic samples were collected (June 2018, 2019) from four sampling regions: Kathmandu, Pokhara, South, and Southwest. Red and blue icons indicate allopathic and ayurvedic pharmacies, respectively, though overlap obscures many sites.

MEl1 Bio PAD protoype with 2018 allopathic samples. Each square was loaded with CML282::pCHAMP18; pCHAMP11 (*MEL1* reporter gene) yeast in hydrogel. Cells are not lysed as the yeast secrete MEL1.

A and B: Lac Z Bio PADs with 2018 ayurvedic samples. Each square was loaded with CML282::pCHAMP18(human glucocorticoid receptor); pCHAMP10 (gluticorticoid responsive promoter + *LacZ* reporter gene) yeast in hydrogel, sample, and media. X-β-Gal and βME were added after lysing cells (A). Lack of blue in spot 2 (B) indicates a multi-lab issue likely involving both X-α-Gal and X-β-Gal sugars as both *LacZ* and *MEL1* reporter genes failed to produce blue color change in the second summer. Lanes on all BioPADs: 1) Negative Control 2) Positive control (prednisone); 3-4 Sample 1; 5-6 Sample 2.
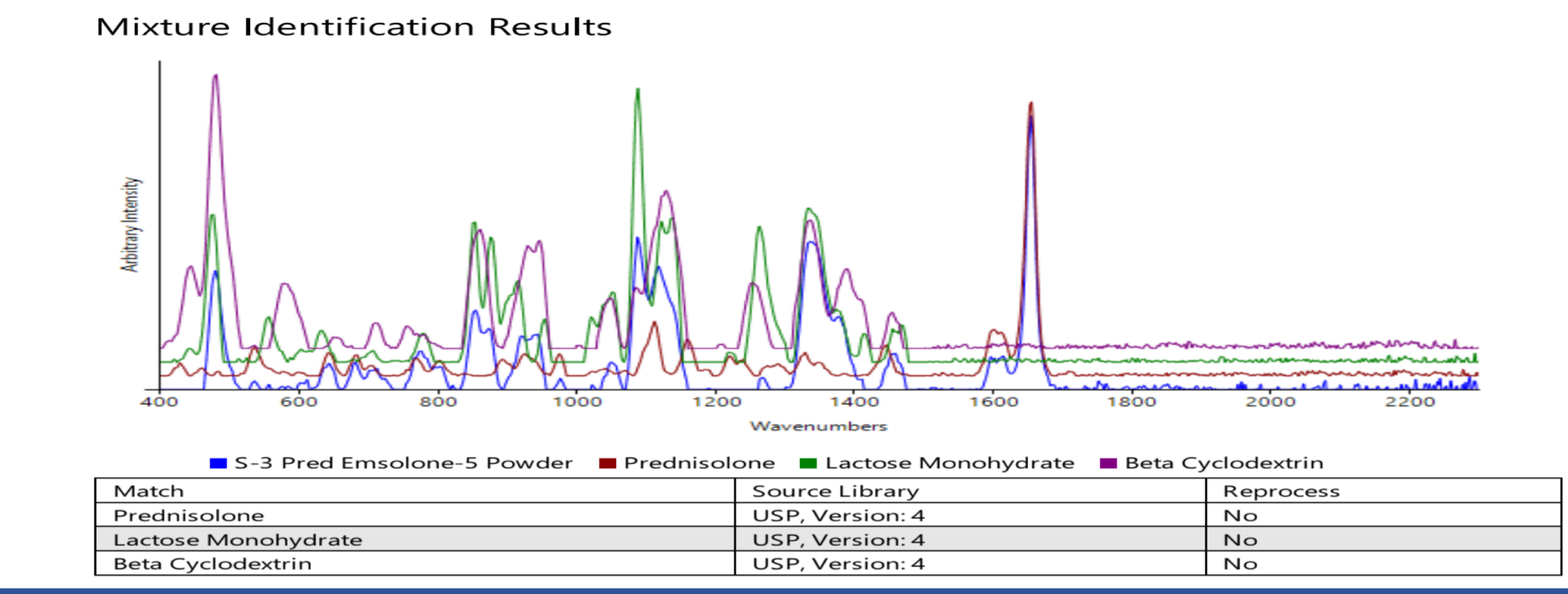

Mira Cal 3.0 Raman spectra for a 5mg prednisolone tablet (80mg total) crushed into powder. Tablets are a mixture match for prednisolone and match with filler lactose monohydrate. The sample also matches with beta cyclodextrin, another type of glucocorticoid. Raman SERs could be used to gain a more accurate spectral analysis, however it would increase costs for field tests.
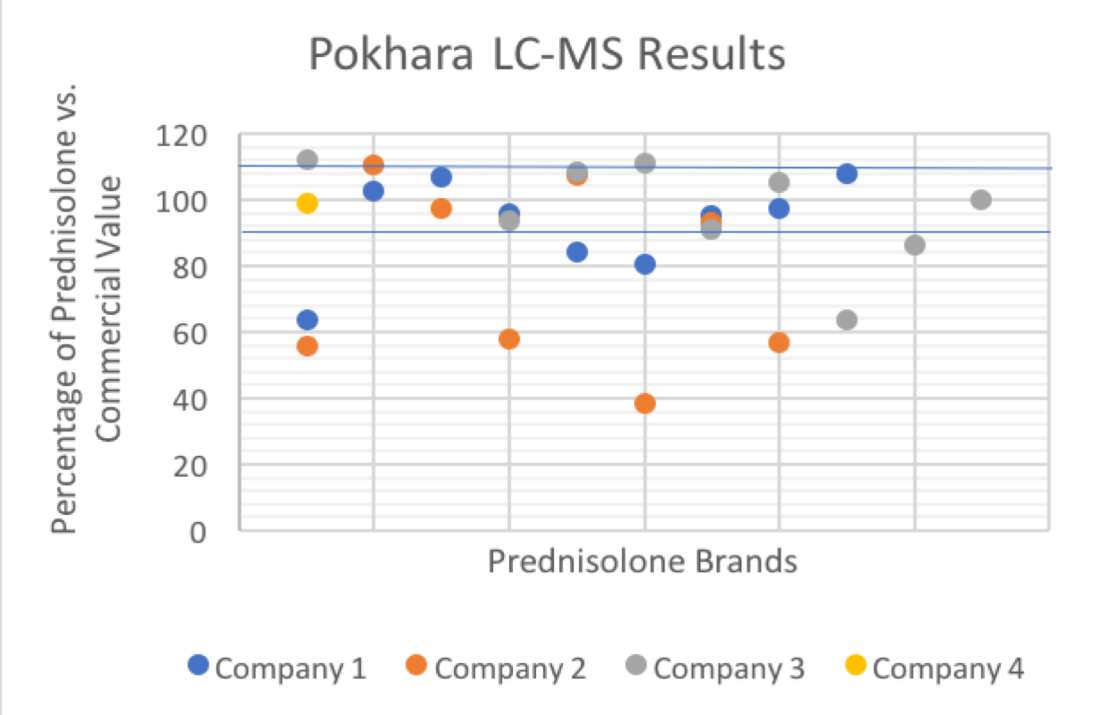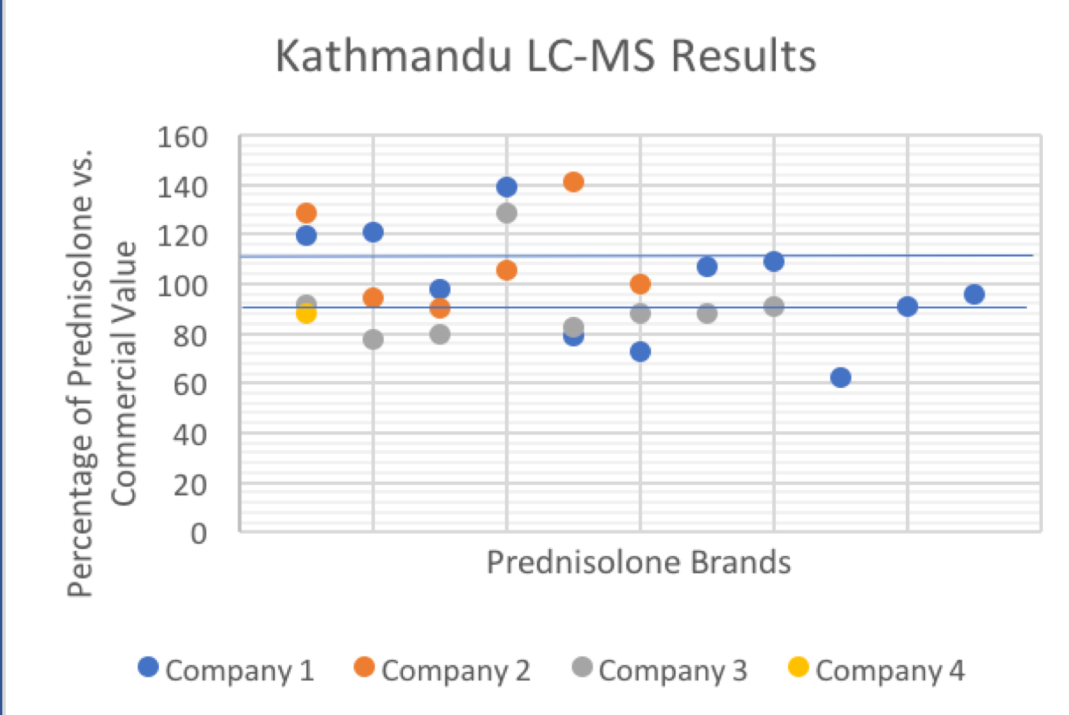
Region	Medicines with prednisolone match (%)	Medicines with no prednisolone match (%)	Medicines with no glucocorticoid steroid match (%)
KTM	68.00(T) 96.00(C)	32.00(T) 4.00(C)	12 (T) 0 (C)
POK	79.31(T) 72.41(C)	20.69(T) 27.59(C)	13.79 (T) 20.69(C)
South	78.79(T) 69.7(C)	21.21(T) 30.3(C)	12.12(T) 9.1(C)
SW	93.55(T) 80.65(C)	6.45 (T) 19.35(C)	3.23 (T) 9.7(C)

Raman spectra for allopathic tablets (T) or crushed pills (C) (2019 samples). Tablets were crushed to ensure pill was not matching coating compounds. Using WHO sampling guidelines, each region had ~30 allopathic samples collected and subjected to Raman Spectroscopy. Coatings do not appear to be present, but pills not evenly mixed. Raman Spectra suggest 0-21% of allopathic tablets from each region contain low/undetectable levels of prednisolone.
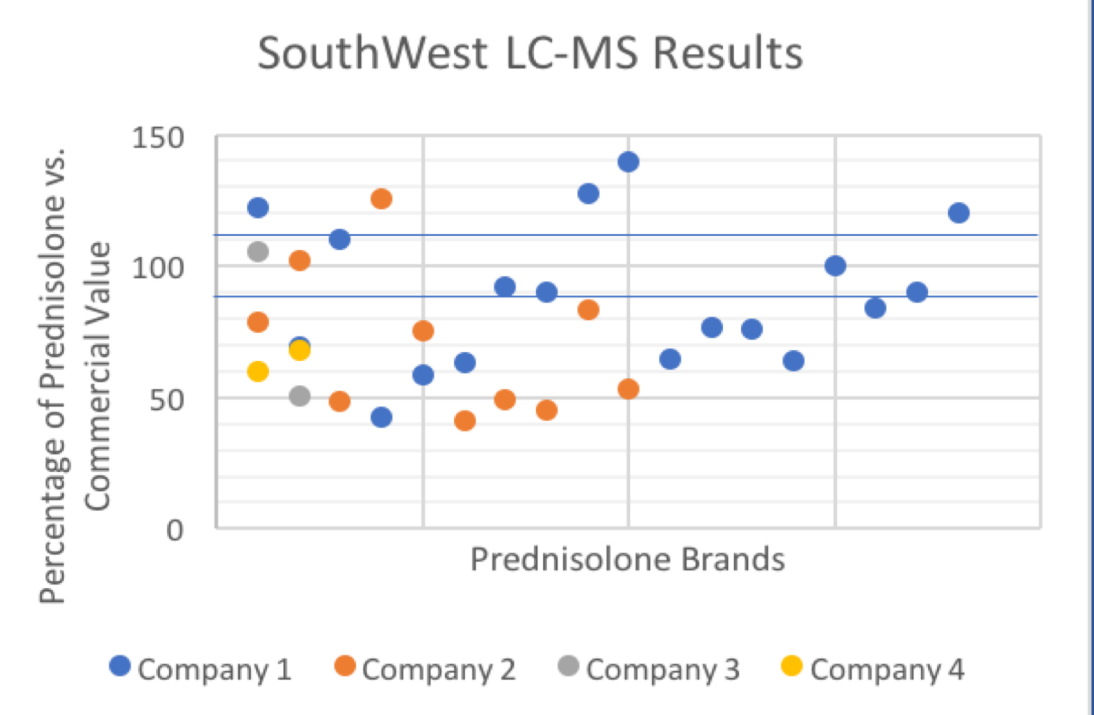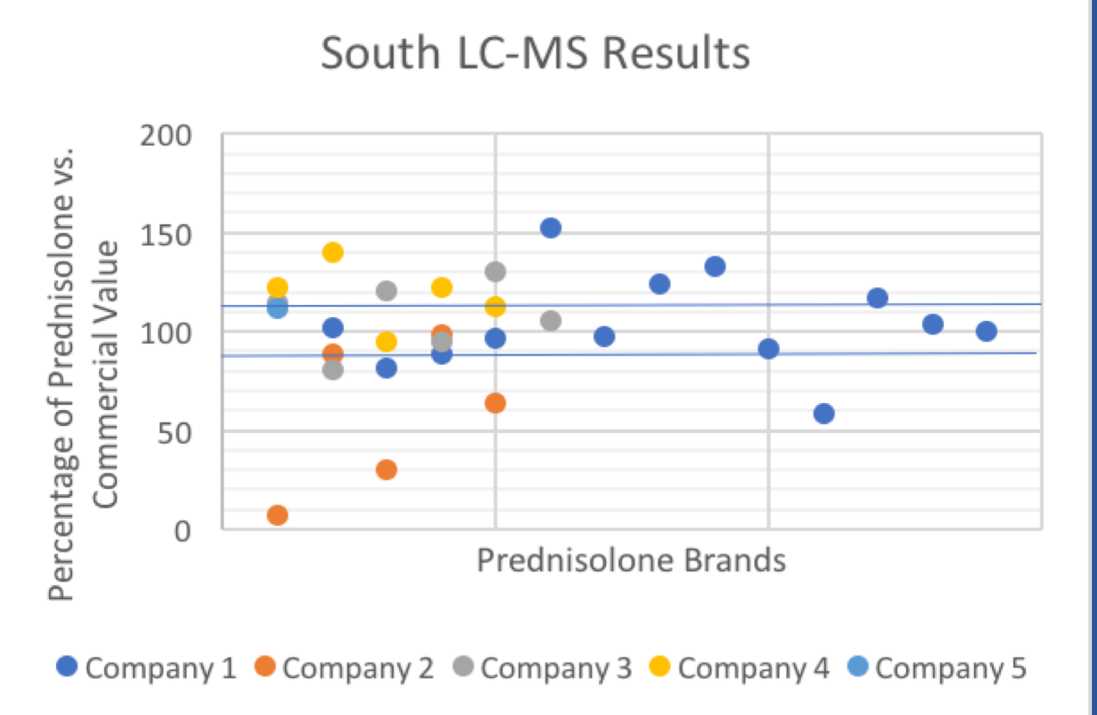
Liquid Chromatography – Mass Spectrometry analysis for all four regions. The different colors represent the different Nepali (1-4) and Indian (5) brands of prednisolone medicine. The two blue lines indicate the US pharmacopeia standards for dosage of pharmaceuticals (within 90-110% of commercial value).

Discussion

- Bio PADs could not be used for comparison to the other methods because PADs in multiple labs had problems with the lack of color change, possibly due to problems with the X-gal sugar. Embedding sugars in paper may solve the problem, but further testing is required
- LC-MS analysis indicated potential under dosing of active pharmaceutical ingredient (API) primarily in the Southwest region, where 20/32 samples had calculated doses under the US standard
- LC-MS did not indicate a difference between under dosing by a specific company
- Comparisons between individual samples don't indicate an agreement between Raman spectra and LC-MS analysis. Raman spectra with no glucocorticoid steroid match appear to have LC-MS analysis with prednisolone levels that range from overdosed to under dosed
- Tablet or crushed forms did not appear to increase accuracy of Raman spectra as compared to LC-MS analysis
- As prednisolone is a mixture (5-20 mg API per ~80 mg pill), Raman spectrometry without the more expensive SERs addition is not recommended. Data comparing Raman to LC-MS analysis confirmed this prediction.
- Raman SERs should be used for future analysis

Future Directions

- LC-MS: Test allopathic from 2018 and ayurvedic medicines 2018 and 2019
- Improve Bio PAD accuracy with X-gal stabilization. Test all samples.
- Raman: Test all samples using the more expensive Raman-SERs ($8 increase per test)
- KIAS will be used to communicate any confirmed problems to the Nepali authorities.

References
1. Gatehouse, J. 2019. Counterfeit medicines, from headache pills to cancer drugs, have become a multibillion-dollar problem. *CBC News: The National Today*.
2. World Health Organization. Substandard and Falsified (SF) Medical Products (January 2018).
3. Barbiere, C. 2017. Counterfeit Chinese and Indian drugs invade Africa. *EURACTIV Network*
4. Weaver, A.A., et al. 2015. Incorporating yeast biosensors into paper-based analytical tools for pharmaceutical analysis. *Anal. Bioanal.* 407: 615-619.
5. World Health Organization. Recommendations on the content of a survey protocol for surveys of the quality of medicines (July 2014).
6. Belli G. et al. 1998. An activator/repressor dual system allows tight tetracycline-regulated gene expression in budding yeast *NAR* 26: 942-7.
7. Miller, C. et al. 2010. Single Plasmids Expressing Human Steroid Hormone Receptors and a Reporter Gene for Use in Yeast Signaling Assays. *PMC* 63:73. (obtained from addgene).
8. Clontech Laboratories, Inc. (now Takara Bio, USA, Inc) Mountain View, CA, USA.
9. Keppler-Ross et al. 2008. A new purple fluorescent color marker for genetic studies in *Saccharomyces cerevisiae* and *Candida albicans. Genetics* 179(1):705-10