

Comparative Studies of the Effects of Two Novel Sugar Drug Candidates on the CYP 1A2 and CYP 2E1 Enzymes in Different Sexed Rats Using a “Cocktail” Approach

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Abstract: The sex-based differences between the effects of two novel sugar-based drug candidates, a sulfated polymannuroguluronate (SPMG-911) and an acidic oligosaccharide sugar chain compound (AOSC-971), on the enzymes CYP 1A2 and CYP 2E1 were investigated. The results showed that neither SPMG-911 nor AOSC-971 have any effect on CYP1A2, while AOSC-971 induced the CYP 2E1 in male rats. The results are useful for their safety evaluation, as well as for the prediction of inter-drug interactions associated with the two drugs.

Keywords: Cocktail approach, enzymes, drug safety evaluation.

Introduction

Sulfated polymannuroguluronate SPMG-911, a novel sulfated polysaccharide rich in 1-4 linked β -D-mannuronate, prepared by sulfate modification of an alginate extract from brown algae, possesses a certain 1,4-linked β -D-mannuronate to α -L-guluronate ratio and an average molecular weight of 10KD. Our previous work has demonstrated a significant inhibition of the replication of HIV and SIV by SPMG-911 both *in vitro* and *in vivo*.

The acidic oligosaccharide sugar chain compound AOSC-971, a novel marine-derived acidic oligosaccharide, was extracted from brown algae *Echlonia Kurome Okam* by enzymatic

depolymerization. With an average molecular weight at 1300 Da., its primary sequence is rich in β -D-mannurinic acid linked by 1-4 bonds endowing the structure with a negative charge.

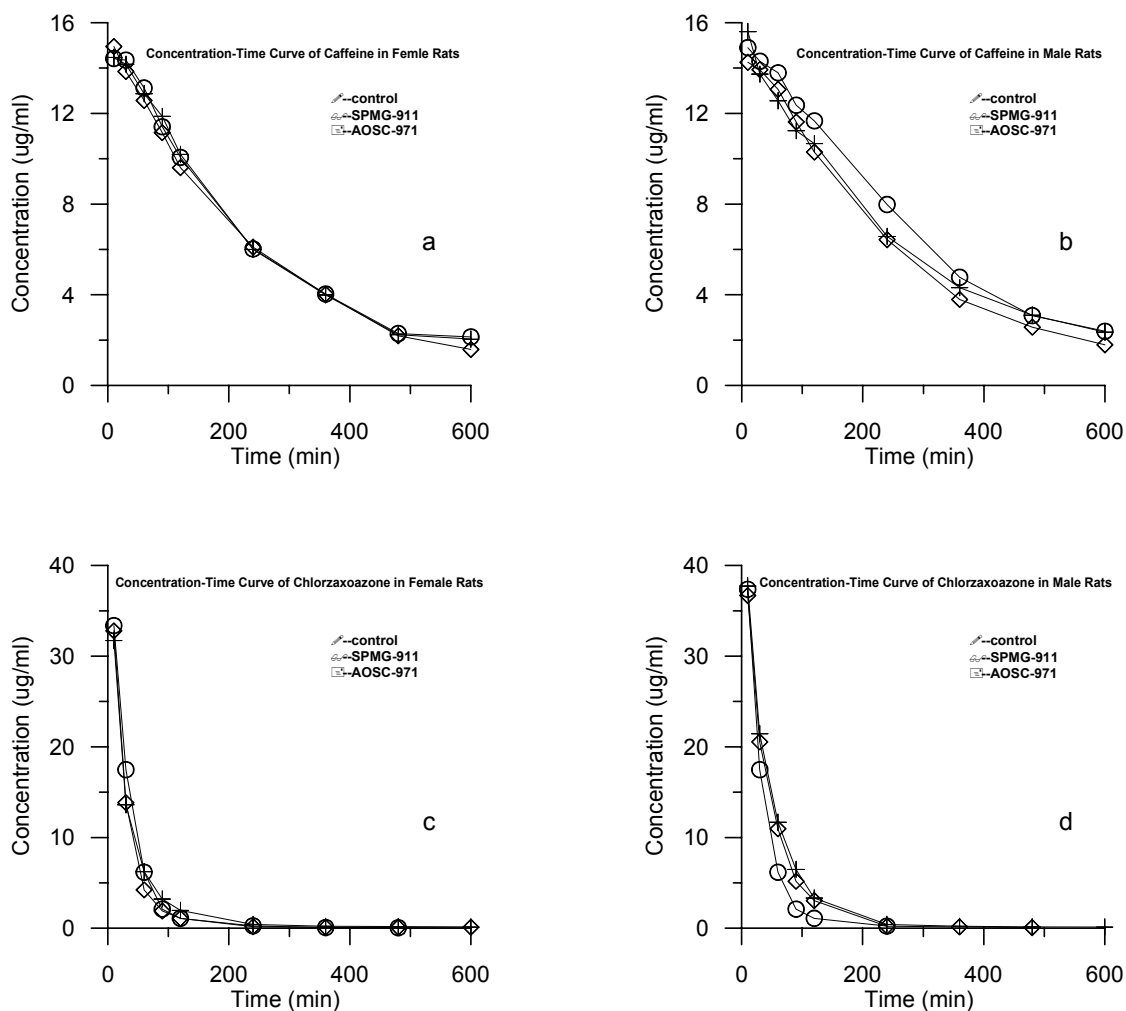
This study was related to a safety assessment of SPMG-911 and AOSC-971, as well as the prediction of interdrug interactions associated with them by investigating their influences on CYP 1A2 and CYP 2E1 enzymes, especially their sex-based differences, through comparison of pharmacokinetics data of caffeine and chlorzoxazone, which are the special “cocktail” probe drugs for CYP 1A2 and CYP 2E1, respectively [1, 2].

Results

Concentration-time curves of the two probe drugs

Rats in each group were injected with the caffeine and chlorzoxazone probe drugs and the concentrations of the samples were determined using the methodology described in the Experimental section. Results are shown in Figures 1a-d.

Figure 1. Concentration-Time Curves of: a) caffeine in female rats; b) caffeine in male rats; c) chlorzoxazone in female rats; d) chlorzoxazone in male rats.



Pharmacokinetic data of the two probe drugs

The caffeine and chlorzoxazone concentrations were least squares fitted with different models using the 3P87 software. With the weights being $1/C/C$, the caffeine and chlorzoxazone results were found to agree with the single and double compartment model, respectively. The pharmacokinetics data of caffeine and chlorzoxazone are given in Tables 1 and 2, respectively. The results showed that there were obvious differences of $T_{1/2}$ and K_{10} between AOSC-971 and the control group ($P < 0.05$), while no significant differences were found between SPMG-911 and the corresponding control group ($P > 0.05$).

Table 1. Pharmacokinetic Data of Caffeine ($\bar{x} \pm s, n = 5$).

| | $T_{1/2}(\text{min})^*$ | | | | $AUC(\text{ug.min/mL})^*$ | | | | K | | | |
|------------|-------------------------|------|----------|------|---------------------------|------|----------|------|--------|------|---------|------|
| | female | P | male | P | female | P | male | P | female | P | male | P |
| Control | 168.68±6 | | 271.31±1 | | 3771.68± | | 5734.01± | | 0.004± | | 0.004±0 | |
| | 6.69 | | 69.02 | | 1163.06 | | 2288.98 | | 0.002 | | .002 | |
| SPMG-911 | 193.97±9 | 0.63 | 165.31±5 | 0.30 | 4378.54± | 0.49 | 3562.22± | 0.22 | 0.004± | 0.92 | 0.005±0 | 0.49 |
| | 1.68 | | 0.04 | | 1447.58 | | 834.07 | | 0.001 | | .002 | |
| AOSC - 971 | 255.22±1 | 0.21 | 247.26±7 | 0.80 | 4954.75± | 0.34 | 5240.00± | 0.76 | 0.003± | 0.48 | 0.003±0 | 0.56 |
| | 26.89 | | 4.82 | | 2324.51 | | 1188.43 | | 0.002 | | .001 | |

Table 2. Pharmacokinetic Data of Chlorzoxazone ($\bar{x} \pm s, n = 5$).

| | $T_{1/2}(\text{min})^*$ | | | | $AUC(\text{ug.min/mL})^*$ | | | | K_{10} | | | |
|------------|-------------------------|------|----------|------|---------------------------|------|-----------|------|----------|------|---------|------|
| | female | P | male | P | female | P | male | P | female | P | male | P |
| Control | 177.73± | | 271.82±1 | | 2051.42±1 | | 1834.50±1 | | 0.034± | | 0.025± | |
| | 103.30 | | 74.86 | | 397.32 | | 837.4 | | 0.012 | | 0.014 | |
| SPMG-911 | 261.34± | 0.43 | 324.67±2 | 0.71 | 1170.77±3 | 0.28 | 1283.46±4 | 0.53 | 0.040± | 0.34 | 0.042± | 0.19 |
| | 201.75 | | 57.31 | | 09.46 | | 54.00 | | 0.005 | | 0.014 | |
| AOSC - 971 | 185.37± | 0.93 | 72.43± | 0.04 | 1060.49±3 | 0.25 | 1548.31±5 | 0.75 | 0.037± | 0.66 | 0.0429± | 0.04 |
| | 162.54 | | 34.28 | | 77.66 | | 19.39 | | 0.011 | | 0.0082 | |

* $T_{1/2}$ = Half Life of Elimination; AUC = Area Under Curve

Discussion

Cytochrome P450 (CYP 450), a supergene family, consists of many isozymes which are involved in biotransformation of many drugs, exogenous substances and precarcinogens and it plays a very important role in drug metabolism.

CYP450 can be induced or inhibited by some drugs, the activities or toxicities of which therefore can be affected accordingly. In fact, both CYP 1A2 and CYP 2E1 play critical roles in the metabolism of drugs because of their wide distribution and high concentration *in vivo*. Studies of drugs' effects on these two enzymes are very useful for drug safety evaluations, as well as the prediction of inter-drug interactions and the design of more suitable drug administration protocols.

In this study, SPMG-911 and AOSC-971 exhibited different effects on CYP 450 isomers. With regards to CYP 1A2, SPMG-911 and AOSC-971 showed no effects, as indicated particularly by the pharmacokinetic data of caffeine in the SPMG-911-treated and AOSC-971-treated groups, which displayed no obvious differences compared with the control group. As for CYP 2E1, SPMG-911 has no effect on it, while AOSC-971 shows a potent induction in the male rat. It is a known fact that the induction or inhibition on CYP 450 isomers by some drugs is highly structure dependent. Growing evidence has highlighted that the variations in molecular skeletons characterized by differences in spatial configuration, steric hindrance and even their physical and/or chemical properties, including hydrophobicity or hydrophobicity, etc., likely play important roles in modulating enzyme activities [3-8]. Compared to AOSC-971, SPMG-911 contains different sugar sequences with different molecular weights and different modified residual groups. These differences, at least in part, help us understand the different action of SPMG-911 and AOSC-971 on the CYP 450 isomers. The results observed with AOSC-971 are consistent with the theory that CYP 2E1 is induced by many low molecular weight organic compounds [9,10]. Elucidation of the exact mechanism(s) of action, of course, will require further study.

Conclusions

In summary, our data demonstrated that the influence of AOSC-971 on CYP 450 subfamily enzymes is sex dependent. This sex-based difference should be carefully considered when these compounds are used in future experiments. Furthermore, the combined usage of AOSC-971 with some other drugs, particularly CYP 2E1 inducing drugs, should be carefully considered. As for SPMG-911, there appears to be no such limitation in its combined usage with other CYP450-inducing drugs.

Experimental

Reagents

The dry caffeine and chlorzoxazone powders used as the standards were obtained from The Second Chemical Reagent Factory (Shanghai, China) and the Medicine and Drug Industry Institute (Shandong, China), respectively. Antipyrine (the internal standard) was obtained from the KeMiOu Chemical Reagent Development Centre (Tianjin, China). Drug candidates SPMG-911 and AOSC-971 were purified by the Marine Drug and Food Institute of Ocean University of China (Qingdao, China). Methanol (LUDU Chemical Reagent Factory of Shanghai, China), acetonitrile (HIL, USA) and ammonium hydroxide (First Chemical Reagent Factory of Tianjin, China) were all HPLC grade.

Administration [11]:

30 Wistar rats weighing 230 ± 20 g, half male and half female, were supplied by the Drug Institute of Tianjin. All rats were housed in a room maintained at 23°C and they were allowed access to food and water *ad libitum*. They were randomly divided into six groups with five rats in each group: I) male-SPMG, II) female-SPMG, III) male-AOSC, IV) female-AOSC, V) male-blank, and VI) female-blank. Rats in groups I & II, III & IV, V & VI, were given i.g SPMG-911 and AOSC-971 (both were dissolved with distilled water just before usage) and 0.9% NaCl every morning at a dosage of 85 mg/kg, 360 mg/kg, and 1 mL/kg, respectively. This administration was continued for 6 days before the probe drugs caffeine and chlorzoxazone were injected, each at a dosage of 10 mg/kg, into all rats on the morning of day 7.

Sample collection [11]:

Venous plasma samples (0.5 mL each time) were taken in every rat from the orbital sinus at 0, 10 min., 30 min., 1 h., 1.5 h., 2 h., 4 h., 6 h., 8 h., and 10 h. after the injection on day 7, then centrifuged at 3000 rpm for 5 minutes and the upper layer of the plasma was stored at -20°C .

Sample preparation:

Samples (200 μL) were pipetted precisely into a clean tube after thawing, then the internal standard (20 μL , 110 $\mu\text{g}/\text{mL}$) was added, followed by *t*-butyl methyl ether (5 mL). After shaking adequately a portion of the organic layer (3 mL) was transferred into another clean tube and evaporated to dryness under a stream of nitrogen. The nitrogen pressure was regulated appropriately to avoid any loss of the probe drugs. The residue was dissolved in methanol (120 μL), shaken and centrifuged at 6000 rpm for 1 minute before detection.

Chromatographic conditions:

A Waters HPLC instrument (Waters Company, USA) was used in these studies. The C_{18} column (Dikma Technologies Company, China) had a size of 150×4.6 mm with a particle size of 5.0 μm . The temperature of the column was set to 30°C . The mobile phase was 22:8:70 (v:v:v) methanol-acetonitrile-ammonium oxalate (20mmol/L) at a flow rate of 1mL/min. The volume ratio was changed to 40:15:45 after eight min. Each component of the mobile phase was first purified by suction-filtration, and then ultra-filtration for 10 minutes. The wavelength of the UV detector (Waters Company, USA) was set to 280 nm.

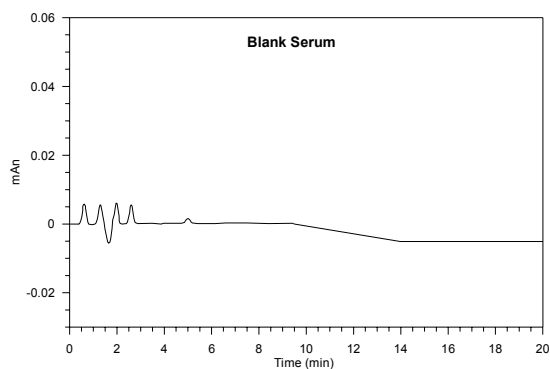
*Assessment of the experimental methods**Selectivity*

Figures 2a-c represent chromatograms obtained from blank serum, serum with probe drugs added *in vitro*, and serum with probe drugs injected *in vivo*, respectively, as described above. Caffeine, chlorzoxazone and the internal standard gave rapidly eluting, fully resolved and sharp symmetrical

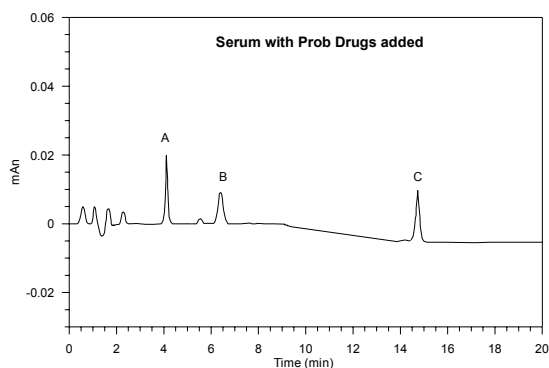
peaks on the chromatograms at retention times of 4.1 min., 6.8 min. and 14.9 min., respectively, both *in vitro* and *in vivo*. No endogenous interfering peaks were found in the blank serum. These results suggest that this method has a high selectivity in detecting the serum concentrations of caffeine and chlorzoxazone.

Figure 2. Chromatograms of probe drugs in different circumstances.

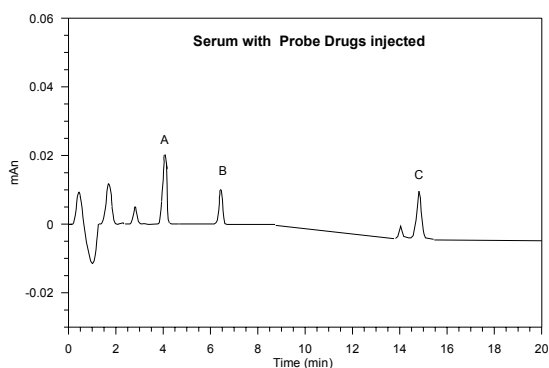
A) caffeine; B) antipyrine; C) chlorzoxazone.



a) Chromatogram of blank serum



b) Chromatogram of serum with probe drugs added.

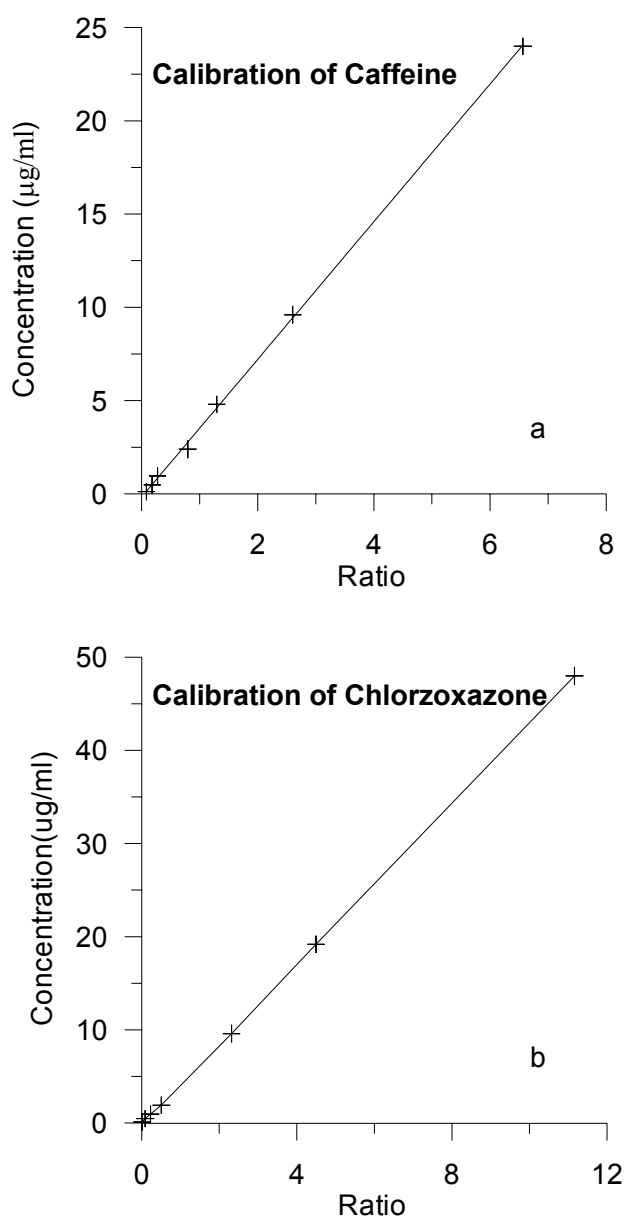


c) Chromatogram of serum with probe drugs injected

Calibration curves

Caffeine and chlorzoxazone were weighed precisely before dissolving and diluting to a 1 mg/mL solution with the HPLC grade methanol. Calibration solutions A–G were obtained by mixing different volumes of the caffeine and chlorzoxazone preparation solutions and 20 μL internal standard (110 $\mu\text{g}/\text{mL}$), then adding blank mice serum to the mark, so that the final caffeine concentrations were 0.12 (A), 0.48 (B), 0.96 (C), 2.4 (D), 4.8 (E), 9.6 (F) and 24 (G) $\mu\text{g}\cdot\text{mL}^{-1}$, respectively, and the chlorzoxazone concentrations were 0.12 (A), 0.48 (B), 0.96 (C), 1.92 (D), 9.6 (E), 19.2 (F) and 48 (G) $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. The corresponding chromatograms were obtained following the procedures described above

Figure 3. Calibration Curves for a) Caffeine b) Chlorzoxazone .



Figures 3a and 3b show the calibration curves of caffeine and chlorzoxazone. The corresponding data, listed in Tables 3 and 4, shows that both compounds display a good linear relationship within the abovementioned concentration ranges (the adopted minimum concentration is less than 1/20 of the maximum for both probe drugs). The respective calibration curve equations are:

$$\text{for caffeine: } Y = 3.689973405 * X - 0.168763287 \quad (n=7, r=0.9995)$$

$$\text{for chlorzoxazone: } Y = 4.027386226 * X + 0.0901732 \quad (n=7, r=0.9934)$$

Table 3. Caffeine Calibration Curve Data.

| Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) | Ratio |
|--|--------|
| 0.12 | 0.0820 |
| 0.48 | 0.1822 |
| 0.96 | 0.2752 |
| 2.4 | 0.7964 |
| 4.8 | 1.2941 |
| 9.6 | 2.6035 |
| 24 | 6.5665 |

Table 4. Chlorzoxazone Calibration Curve Data.

| Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) | Ratio |
|--|----------|
| 0.12 | 0.007406 |
| 0.48 | 0.08978 |
| 0.96 | 0.2274 |
| 1.92 | 0.5048 |
| 9.6 | 2.3189 |
| 19.2 | 4.4944 |
| 48 | 10.1617 |

Intraday and interday reproducibility

The results for caffeine are summarized in Table 5. The RSD for the intraday reproducibility ranged from 3.3 to 7.7% while that of the interday reproducibility ranged from 0.4 to 8.3%. All deviations were within 10%.

Table 5. Intraday and Interday Reproducibility of Caffeine (mean \pm SD, n=3.)

| Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) | Intraday reproducibility | | Interday reproducibility | |
|---|---|------------|---|------------|
| | Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) | RSD (%) | Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) | RSD (%) |
| 0.12 | 0.13 \pm 0.01 | 7.7 | 0.12 \pm 0.01 | 8.3 |
| 4.8 | 4.61 \pm 0.15 | 3.3 | 4.53 \pm 0.25 | 5.5 |
| 24 | 24.06 \pm 1.50 | 6.2 | 22.34 \pm 0.08 | 0.4 |

The chlorzoxazone results are summarized in Table 6. The RSD for the intraday reproducibility ranged from 0.8 to 7.8% while that of the interday reproducibility ranged from 1.8 to 4.3%. All deviations were also within 10%.

Table 6. Intraday and Interday Reproducibility of Chlorzoxazone (mean \pm SD, n=3.)

| Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) | Intra-day reproducibility | | Inter-day reproducibility | |
|---|---|------------|---|------------|
| | concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) | RSD (%) | concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) | RSD (%) |
| 0.12 | 0.12 \pm 0.001 | 0.8 | 0.113 \pm 0.002 | 1.8 |
| 9.6 | 9.43 \pm 0.31 | 3.3 | 9.29 \pm 0.40 | 4.3 |
| 48 | 41.69 \pm 3.24 | 7.8 | 44.52 \pm 1.21 | 2.7 |

Detection limits

The limit of detection of this method was defined at a signal-to-noise ratio of 3:1, so that of caffeine was 0.05 $\mu\text{g}\cdot\text{mL}^{-1}$ while for chlorzoxazone it was 0.1 $\mu\text{g}\cdot\text{mL}^{-1}$.

Extraction rates

The results of the extraction rates of caffeine and chlorzoxazone are given in Tables 7 and 8, respectively. The extraction rates at different concentrations were all above 70%.

Table 7. Extraction Rate of Caffeine (mean \pm SD, n=3).

| Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) | Detected Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) | Rate (%) |
|---|--|-------------|
| 0.12 | 0.13 \pm 0.01 | 108.3 |
| 4.8 | 4.61 \pm 0.15 | 96.04 |
| 24 | 24.06 \pm 1.50 | 100.25 |

Table 8. Extraction Rate of Chlorzoxazone (mean \pm SD, n=5).

| Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) | Detected Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) | Rate (%) |
|---|--|-------------|
| 0.12 | 0.12 \pm 0.001 | 100 |
| 9.6 | 9.43 \pm 0.31 | 97.34 |
| 48 | 41.69 \pm 3.24 | 86.85 |

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Sample Availability: Available from the authors.