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Urine and Serum Analysis of Consumed Curcuminoids Using an IκB-Luciferase Surrogate Marker Assay

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Abstract. *Background: Curcumin metabolites are detectable in body fluids such as serum and urine. We have developed a novel assay that can detect metabolites in such body fluids by measuring their effect on the nuclear factor kappa B/inhibitor of kappa B (NF-κB/IκB) pathway. Patients and Methods: Fifteen healthy individuals were enrolled in the study and randomly assigned to two groups: control group (five) and curcumin group (ten). The test group ingested 8 g of the curcuminoids (C^3-Complex™) with 16 oz of bottled water. Blood and urine were collected at 0, 4, 8, and 24 h after ingestion. Degradation of the NF-κB/IκB complex was detected by the Genetic Expression and Measurement (GEM™) assay using HCT116 cells stably transfected with PGL3-IκB firefly luciferase. Results: Using our novel GEM assay, the five controls who had not taken curcumin were identified. Conclusion: The GEM assay is a very sensitive and accurate non-invasive assay that could be utilized to detect metabolites in body fluids. It could also serve as a tool to determine participants' compliance during clinical research studies.*

Curcumin is the active ingredient of turmeric, a food spice and colorant widely used throughout India and Southeast Asia. Curcumin is a major constituent of curry powder, to which it imparts its characteristic yellow color. For over 4,000 years, curcumin has been used in traditional Asian and African medicine to treat a wide variety of ailments (1). In this context, we have described a novel approach for determining the metabolic scheme of curcumin in human and mouse liver microsomal preparations using a hybrid quadrupole linear ion trap mass spectrometer coupled with liquid chromatography for the detection of new metabolites (2). In that study, we identified curcumin metabolites that had not previously been reported, such as curcumin bisglucuronide and *O*-demethylated derivatives. In addition, we determined that very similar metabolic pathways of curcumin were observed in human and mouse microsomes (2).

Curcumin consumption does not appear to have a significant effect on the serum lipid profile, unless the absorbed concentration of curcumin is considered, in which case curcumin may modestly increase cholesterol (3). Spectroscopic evidence has shown that curcumin binds to duplex DNA and to RNA bases as well as to the backbone phosphate group, but no conformational changes were observed upon curcumin interaction with these biopolymers (4).

Experimental research has shown curcumin to be a highly pleiotropic molecule capable of interacting with numerous molecular targets involved in inflammation. Based on early cell culture and animal research, clinical trials indicate that curcumin may have potential as a therapeutic agent in diseases such as inflammatory bowel disease, pancreatitis, arthritis, and chronic anterior uveitis, as well as certain types of cancer (5). Extensive experimental and clinical evidence has also indicated that nuclear transcription factor-κB (NF-κB) is activated during the processes of angiogenesis, inflammation and carcinogenesis (6-9), thereby linking them.

The inhibitory effects of curcumin on major inflammatory mechanisms involving cyclooxygenase-2 (COX-2), lipoxygenase (LOX), tumor necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ), NF-κB along with its unrivaled safety profile suggest that curcumin has bright prospects in the treatment of inflammatory diseases (10, 11). Furthermore, we recently demonstrated that curcumin is a potent inhibitor of angiogenesis in the chick chorioallantoic membrane model (12).

The tolerance of curcumin in humans in high single oral doses appears to be excellent (13). Curcumin is absorbed after oral dosing in humans and can be detected as glucuronide and sulfate conjugates in plasma (14).

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Participant compliance in clinical research settings is often difficult to monitor. It is usually monitored through interviews, 'pill counts', and diaries. In studies of standard single drugs, levels of the drugs in blood and/or urine are routinely collected for pharmacokinetic evaluations and confirmation of compliance. However, with complex substances, such as food substances or other herbal products used in complementary and alternative medicine (CAM), there is often an inability to conduct 'classic' pharmacological studies. This is because natural products contain more than one active ingredient making it extremely difficult to determine the pharmacokinetics as opposed to typical drugs that contain one active ingredient. Some complex natural products, however, may lend themselves to more standard approaches. For example, some highly pigmented substances can be detected in both blood and urine following ingestion, and this may allow their traceability by non-invasive monitoring of the urine.

The various considerations described above led us to design a pilot study, presented in this report, to evaluate the feasibility of detecting a correlation between ingested curcuminoids and TNF-α-induced activation by detecting degradation of the curcuminoid-targeted NF-κB/IκB complex in the blood and urine following ingestion. If shown to be feasible, the assay described in this study may have potential as a research tool to monitor an individual's compliance in long-term clinical research studies.

Table I. *Detection of metabolites in urine using GEM assay.*

Participant	Time of sample (h)			
no.	0	4	8	24
1	–	+	–	–
2	–	+	–	–
3	–	+	–	–
4	–	–	–	–
5	+	+	+	–
6	–	+	+	+
7	–	+	–	–
8	+	–	–	–
9	–	+	–	–
10	–	+	+	–
11	+	+	–	–
12	–	+	+	–
13	+	+	–	–
14	+	+	+	–
15	–	+	–	–
16	–	+	+	–
17	+	–	–	–
18	–	+	+	–
19	–	–	–	–
20	–	+	+	–
21	–	+	–	–
22	–	+	–	–
23	+	–	–	–
24	–	+	–	–
25	+	+	–	–

Patients and Methods

Reagents. Curcumin (C3-Complex™) was purchased as an acetone-extracted orange-yellow crystalline powder from Sabinsa Corporation (East Windsor, NJ, USA). The curcumin complex used in this study contained more than 95% curcuminoids as analyzed by HPLC. The curcuminoids comprised of 79.86% curcumin, 17.30% demethoxy curcumin, and 2.84% bisdemethoxycurcumin. The content of total heavy metals (lead, cadmium, arsenic, and mercury) was 10 μg/g.

Study participants. This pilot clinical trial was conducted as an open-label study. Fifteen eligible volunteer participants (males and females) were enrolled at a 1:1 ratio and had normal organ function. Although minorities were sought, no attempt was made to stratify by race, due to the small pilot nature of this study. The participants were 18 years of age or older and were randomly assigned to one of two test groups: a negative control group (n=5) that did not receive curcuminoids and a test group (n=10) that received curcuminoids. All participants signed informed consents, and fasted for 48 h from foods containing agents that can result in degradation of the NF-κB/IκB complex. Eight grams of the curcuminoid C^3-Complex powder (referred to as C^3 hereafter) were taken orally with the aid of 473 ml of bottled water (Ozarka, Wilkes Barre, PA, USA). The curcuminoid powder was administered and the specimens collected at the General Clinical Research Center, University of Oklahoma Health Sciences Center, Oklahoma City.

Sample collection. Blood and urine samples were collected prior to sample ingestion (0 h) and at 4, 8, and 24 h post sample ingestion and placed on ice. Plasma was prepared from the blood samples by gentle centrifugation, and then plasma and urine samples were divided into small aliquots, logged into the database, and stored at minus 80˚C for later use.

IκB-luciferase degradation assay. Degradation of the NF-κB/IκB complex was detected by the Genetic Expression and Measurement (GEM™) assay using HCT116 cells stably transfected with PGL3-IκB firefly luciferase and referred to as HCT116/IκB-Luc cells. The methodology to generate HCT116/IκB-Luc cells and the principles of the GEM assay have been described elsewhere (15). Briefly, the assay is based on the ability of TNF-α to induce degradation of IκB bound to NF-κB and thus generate active NF-κB, allowing the unbound NF-κB to translocate into the nucleus. The remaining intact IκB can be relatively quantified by monitoring a luciferin fluorochrome bound to IκB. In this study, HCT116 cells were transfected with IκB-Luc (HCT116/ IκB-Luc) and seeded on 96-well plates at a density of 3×10^4 cells/well and allowed to adhere overnight. The cells were then treated with 20 μl of plasma or urine for 2 h followed by treatment with TNF-α for 30 min at 37˚C. After washing with phosphate buffered saline (PBS), cell lysis buffer was added and the absorbance read at 260 nm using a Synergy HT plate reader (BioTek Instruments, Winooski, VT, USA). The results are reported as mean±SEM. All measurements were performed in triplicate. Statistical differences between treatments were evaluated using Student's *t*-test and were considered significant when $p<0.05$.

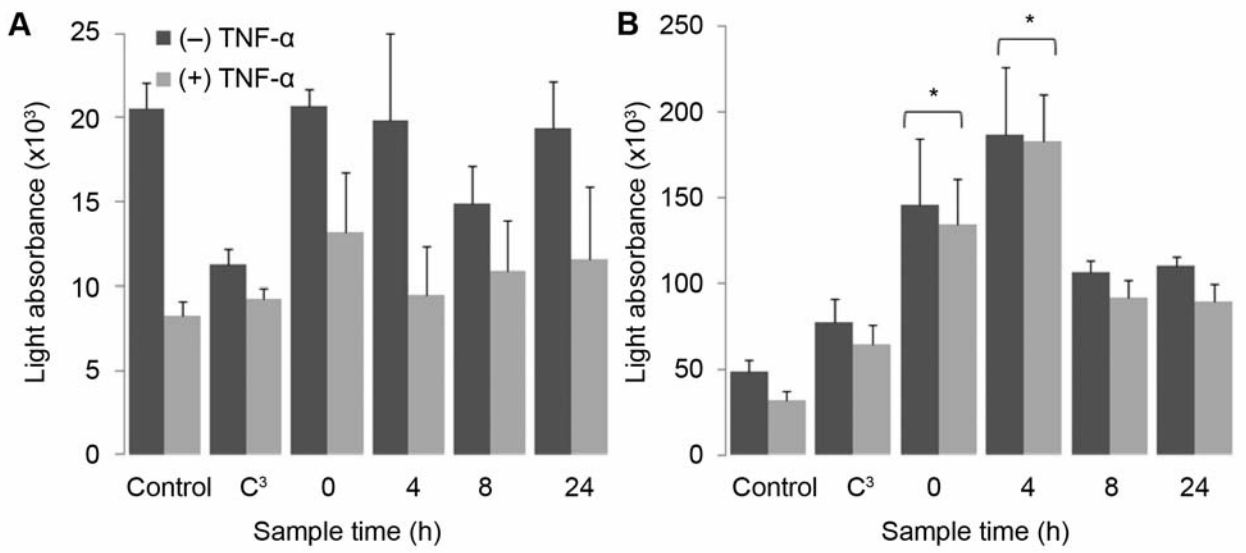

Figure 1. *Urine from individuals 23 and 25 tested in TNF-α mediated degradation/protection of I-κB (GEM assay). Urine samples were collected prior to ingestion (0 h) and then at 4, 8, and 24 h following ingestion of the assigned study product (water or curcuminoids). HCT 116 cells stably expressing IκB-Luc fusion protein were pretreated with urine (20 μl/well) for 2 h followed by the addition of TNF-α, and incubated for 30 min. Lysates were prepared and the luciferase activity measured using a BioTek plate reader. A: Urine from subject #23 did not significantly protect the IκB-Luc protein from TNF-α mediated degradation at 4 h, indicating that individual #23 must have been in the control group. B: Urine from subject #25 shows a significant increase in IκB-Luc protection as indicated by the increase in absorbance at 4 h. 'Control' refers to a negative control (untreated cells) whereas 'C3' refers to a positive control as evident by the increase in expression after TNF-α treatment. Measurements are reported as mean±SEM; *p<0.05 as compared to control.*

Results

Table I shows that the 4 h urine samples from all ten individuals who took the curcuminoids were positive for detection (indicated by a '+' symbol) using our novel GEM assay, whereas only a few 8 h samples and only one 24 h sample were positive. Therefore, the metabolite concentration detected in urine peaked at 4 h. Figure 1 shows the GEM assay histograms of urine samples from a representative control (1A) and test (1B) participant. The urine from the test participant showed an absorbance increase at 4 h, which was absent in urine from the control.

As recently shown, the GEM assay is a reliable assay that can detect agents that have a significant effect on the NF-κB/IκB signaling pathway (16). This is evident in the sample of HCT116/IκB-Luc cells that were treated with C^3, which was used as a positive control (Figure 1). After treatment with TNF-α, which is an inducer of IκB degradation, C^3 was able to protect the degradation of IκB as compared to the negative control, which included no treatment.

Of the fifteen participants, we were able to identify the five who took only water, namely participants 4, 8, 17, 19, and 23 (Table I), based on their negative result at 4 h. In addition, none of the samples from these participants were positive at 8 or 24 h.

We noticed that some individuals in both the control and test groups gave positive test results in the 0 h samples. Concurrently, we observed that these individuals' urine samples were darker in color than other samples, especially at t=0. The positive results could be due to the presence of salts and metabolites that interfere with the GEM assay.

For the serum samples, we did not detect the presence of curcuminoids at any of the time points for any individual using the GEM assay. These results were confirmed by HPLC analysis (data not shown). This could be due to the fact that the amounts of curcuminoids in the blood peaked and returned to normal levels before our earliest sampling time point (4 h) post-ingestion. Indeed, others have shown that levels of curcumin in the serum peak at 30-60 min after ingestion (17). Future studies should consider earlier collection of blood.

Discussion

The GEM assay is a qualitative assay that can detect polyphenols that interfere with the surrogate IκB-luciferase construct in cultured human colon cancer cells, HCT116. The GEM assay provides a rapid, easy, and low cost methodology to detect the presence or absence of metabolites in urine and potentially in other body fluids. It could also be used to test participants' compliance with clinical trial procedures. The assay's sensitivity is demonstrated by the small volume of urine (20 μl) required. Furthermore, the GEM assay could be modified to be quantitative if appropriate standard curves were established. Unfortunately, we did not record the total urine volume collected at each time point. Therefore, we are

unable to report the amount of metabolite detected per unit volume. For future clinical trial purposes, one should consider recording the total urine volume after ingestion of food substances comprising polyphenols in order to quantify the amount of polyphenols absorbed and excreted.

It should be noted that all urine samples were blindly analyzed by the GEM assay. All samples were assayed at least three times, and we were able to detect the control individuals. Furthermore, aliquots of two samples of each group (control and C3) were subjected to HPLC analysis to confirm the GEM assay results in urine. HPLC analysis was chosen because it is a well-established, sensitive, and accurate methodology to identify molecules of known chemical structure.

Based on the results of this study as well as on studies by others, we suggest that future urine and blood samples should be collected at 0, 1, 2 and 4 h. This time range should show the peak absorption of curcuminoids in serum and excretion in urine. It is also more convenient for participants in the clinical study.

In conclusion, the GEM assay is a novel non-invasive tool that can accurately detect metabolites in urine and potentially in other body fluids. It can also determine the biological activity of these metabolites on the NF-κB/IκB signaling pathway. The GEM assay has potential for use in determining participant compliance in clinical research studies.

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Authors' Contributions

FGM, JP, SP, and DS participated in the analyses of human samples using XTT cell proliferation, Western blot, CAM assay, and GEM assay experiments, assisted in data analysis. FGM and PP participated in the design of clinical trial and in design of experiments (DoE), and assisted in data analysis and interpretation and in writing the manuscript. RPR, CH, and SA assisted in the DoE, conducting the clinical trial, data analyses, and in writing the manuscript.

Competing Interests

The Authors declare that they have no competing interest.

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