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# HPLC MONITORING OF CURCUMIN IN PREVENTIVE AND THERAPEUTIC TREATMENT OF RATS TO DIMINISH ACUTE INFLAMMATION AND OXIDATIVE STRESS

**CONEAC ANDREI[a], ORĂSAN MEDA SANDRA[b*], CRIŞAN MARIA[a], MARE CODRUŢA[c], DECEA NICOLETA[d], MIHU CARMEN MIHAELA[a], MUREŞAN ADRIANA[d], LAZAR LEORDEAN VIORICA[e], FILIP MIUŢA[f]**

**ASTRACT.** Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6- heptadiene - 3,5-dione] is a phenolic substance derived from the root of the *Curcuma longa* L plant. It is used as antiproliferative, anti-invasive and antiangiogenic agent with anti-inflammatory functions, cancer chemopreventive activity and antioxidant properties.

The aim of the study was to assess curcumin in rat plasma by HPLC with 425 nm UV detection at specified time intervals (15, 30, 60, 90, 120, 150, 180 minutes) in a treatment plan containing Curcumin in order to diminish acute muscular inflammation and oxidative stress.

The concentration of curcumin in rat plasma was evaluated comparatively in a preventive plan (curcumin administration first, then induced inflammation) versus a therapeutic plan (induced inflammation followed by curcumin administration) on 6 adult female Wistar-Bratislava albino rats assigned to three groups.

The HPLC results showed a decrease of curcumin concentration, with time, in all study groups. The statistical analysis (MANOVA) of the obtained data certified that the treatment plan applied to each group influenced the evolution of curcumin concentration in a significant way. A decrease in the amount of curcumin was noticed when inflammation occurred in the body, most probably determined by the accelerated metabolism in the presence of inflammation.

[a] *Iuliu Hatieganu University, Faculty of General Medicine, Histology Department, 8 Victor Babeş str., RO-400012, Cluj-Napoca, Romania*

[b] *Iuliu Hatieganu University, Faculty of General Medicine, Physiopathology Department, 8 Victor Babes str., RO-400012, Cluj-Napoca, Romania,*

[*] *Corresponding author: meda2002m@yahoo.com*

[c] *Babes-Bolyai University, Faculty of Economics and Business Administration, Department of Statistics – Forecasts - Mathematics, 58-60 Teodor Mihali str., RO-400591, Cluj-Napoca, Romania*

[d] *Iuliu Hatieganu University, Faculty of General Medicine, Physiology Department, 8 Victor Babes str., RO-400012, Cluj-Napoca, Romania*

[e] *Vasile Goldis Western of University Faculty of General Medicine,* 86, Liviu Rebreanu, 310045, Arad, *Romania*

[f] *Babeş-Bolyai University, Raluca Ripan Institute for Research in Chemistry, 30 Fântânele str., RO-400294 Cluj-Napoca, Romania*

The study of oxidative stress levels measured by the variables MDA and PC was performed in different rat tissues: muscle, liver *and* kidneys, for each study group and the highest results were registered in the muscle.
The results obtained in both our research directions validated the anti-inflammatory and antioxidant effect of curcumin.

***Keywords:*** *Curcumin, inflammation, HPLC, oxidative stress*

## INTRODUCTION

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6- heptadiene-3,5-dione] is a phenolic substance derived from the root of the *Curcuma longa* L plant [1]. It is the yellow pigment in turmeric or curry powder, commonly known as a dietary Indian spice and a natural coloring agent for food [1,2]. Curcumin is one of the most highly researched phytochemicals because it has been shown to be an antiproliferative, anti-invasive and antiangiogenic agent, which exhibits numerous activities [3,4].

Over the last half century studies focused on the important anti-inflammatory function of curcumin, which is based on the downregulation of inflammatory transcription factors (NF-kB and AP-1), enzymes (COX-1 cyclooxygenase, MMPs or glutathione S-transferases) and important cytokines (tumor necrosis factor, interleukin 1 and 6), adhesion molecules, proliferation (EGFR and Akt) and cell survival proteins and pathways (ß-catenin and adhesion molecules) [2-4]. Curcumin inhibits the activity of various kinases, downregulates cell cycle arrest (cyclin D1, E and MDM2) and, on the other hand, it upregulates the proteins *p21*, *p27* and *p53* [3].

Besides suppressing specific inflammatory factors, following oral and topical administration, curcumin has cancer chemopreventive activities, that also include the activation of apoptotic mechanism by the up-regulation of the caspase family proteins and by the downregulation of anti-apoptotic genes (Bcl-2 and Bcl-XL)( [4-8]. It is also considered a mediator of chemoresistance and radioresistance due to the molecular response of cancer cells to curcumin at genomic level, an aspect which was explored by cDNA microarrays analysis [3,4].

Several articles underlined its antioxidant properties as Curcumin reduces the reactive oxygen species (ROS) (including superoxide anions and hydroxyl radicals) and *diminishes* oxidative damage [9-12]. As a result of its important role in different physiopathological mechanisms, the potential of Curcumin was evaluated in neoplastic, neurological, cardiovascular, pulmonary

and metabolic diseases [2]. Several studies considered Curcumin a therapeutic agent in wound healing, diabetes, Alzheimer disease, Parkinson disease, cardiovascular disease and arthritis. Some clinical trials also showed its benefits in familial adenomatous polyposis, inflammatory bowel disease, ulcerative colitis, colon cancer, pancreatic cancer, hypercholesterolemia, atherosclerosis, pancreatitis, psoriasis, chronic anterior uveitis and multiple mieloma [3-5].

Despite its promising biological effects, some studies reported low systemic bioavailability of curcumin after oral dosing [1,13]. Although investigated so far in both animal models and human subjects in phase I clinical studies, the pharmacodynamics and pharmacokinetics of Curcumin lack important information and the research in the field is still open [2,14].

Several methods have been developed to determine curcumin in biological samples, high performance liquid chromatography (HPLC) with UV detection [1,7,13,15,16], tandem mass spectrometric (MS) detection [17-20] or ultra performance liquid chromatography (UPLC) [21].

The results of similar studies encouraged us to develop our own model and to investigate curcumin therapy on an animal model: Wistar adult rats [22,23,24,25,26,27].

The aim of the study was monitoring curcumin in rat plasma by HPLC with UV detection at specified time intervals (15, 30, 60, 90, 120, 150, 180 minutes) in a treatment plan in order to diminish acute muscular inflammation and oxidative stress. We assessed comparatively the concentration of curcumin in rat plasma in a preventive (first curcumin administration and then induced inflammation) versus a therapeutic plan (induced inflamation followed by curcumin administration). It was also our purpose to evaluate oxidative stress in muscles and some internal organs (liver and kidney) by MDA (Malondialdehyde) and PC (protein carbonyl).

## RESULTS AND DISCUSSION

### HPLC analysis

The initial trials for curcumin determination from plasma were performed based on literature information [1,15]. We developed a simple, rapid and sensitive HPLC method to determine curcumin levels from rat plasma. According to the chemical characteristics, the maximum UV adsorption of curcumin was 425 nm and set as detection wavelength. The mobile phase was optimized for good resolution and symmetric peak shape for analysis, as well as a short run time of 6 min. Thus we designed a mobile phase consisting of a mixture of methanol – 0.1% formic acid (90-10, v/v) and a

Lichrosorb RP-C18 column (25 x 0.46 cm) at 30 ºC column temperature. The flow rate was set at 1.2 mL $min^{-1}$. Because of the complex nature of plasma, a pre-treatment procedure was needed to remove protein and potential interferences prior to HPLC analysis. Thus, methanol was used for the precipitation of proteins from rat plasma.

The linear regression analysis of curcumin was developed by plotting the peak area of curcumin versus the concentration of curcumin in ng $mL^{-1}$. The regression coefficient of calibration curve was $R^2$ = 0.996 within the range 100–2000 ng/mL for curcumin, extracted from rat plasma samples. The linear equation for curcumin was Y = 0.00062 X - 0.0143 (n=15 points). The limit of quantification (LOQ) of curcumin detected in rat plasma was 7 ng $mL^{-1}$.

A 2007 American study described the bioavailability of curcumin in rodents and humans and reported that the amount of free curcumin detected in the serum collected at 1 hour postgavage administration of curcumin in mice, fell below the limit of quantification of 5 ng $mL^{-1}$at HPLC determination [20].

In Figure 1 HPLC chromatograms of blank plasma, curcumin standard and Sample I are presented.

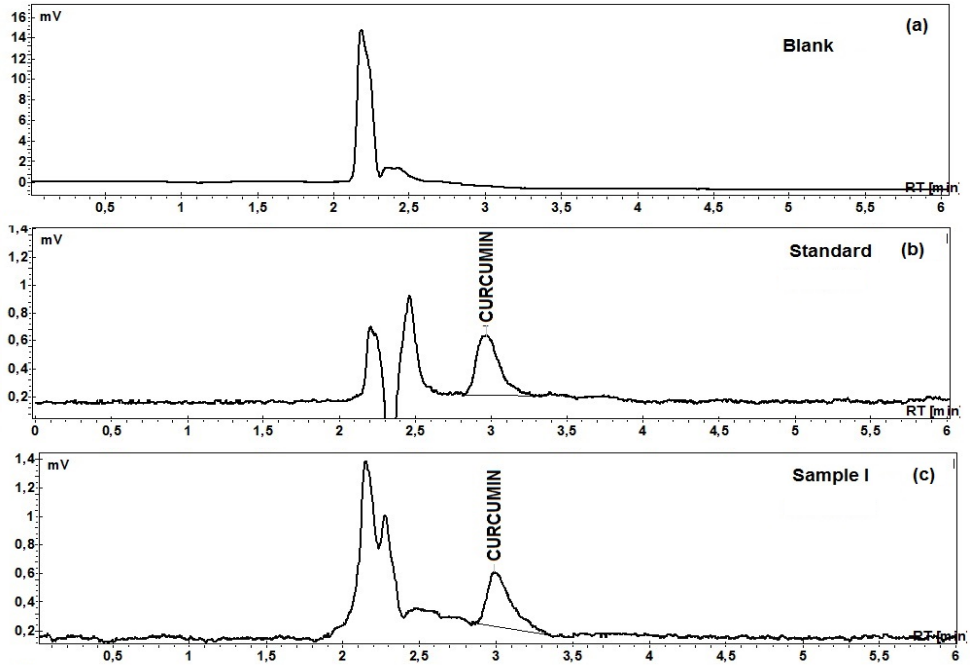


**Figure 1.** HPLC chromatograms **of: (a)** blank plasma. **(b)** standard curcumin at 40ng/mL in blank plasma, expressed as a peak at the retention time of 2,89 min. **(c)** Sample I (rat plasma from Group I obtained at 15 min after oral administration of curcumin suspension of 150 mg Curcumin / rat kg body weight.

The HPLC analysis was performed on plasma samples from different study groups (Groups I-III) collected at 15, 30, 60, 90, 120, 150, 180 minutes. The animals in Group I (Samples I and IB) were treated with curcumin only. Those in Group II (Samples II and IIB) received preventive curcumin followed by induced inflammation, while animals in Group III (Samples III and IIIB) received induced inflammation followed by curcumin treatment.

The HPLC results are included in Figure 2, that displays the concentration of curcumin in the study groups, collected at the specified time intervals. Three HPLC determinations were performed for each sample and the mean value was calculated individually for the mentioned time intervals.

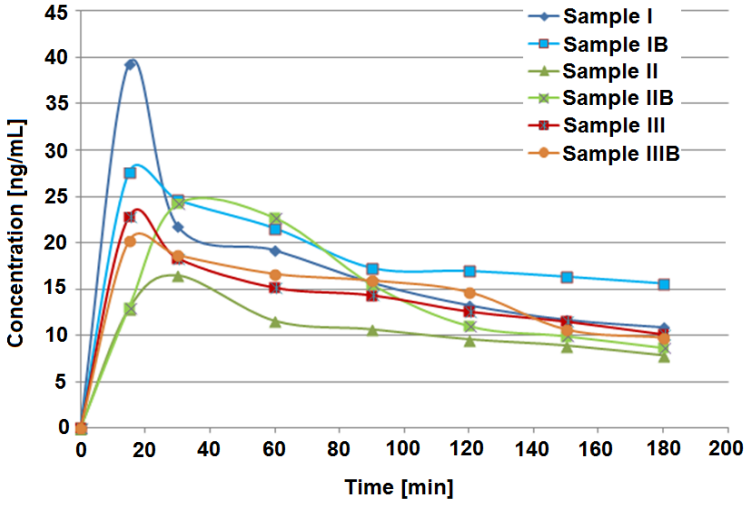


**Figure 2.** HPLC curcumin concentration in plasma samples for Study Groups I-III.

The results obtained showed a decrease of curcumin concentration with time, in the majority of cases, in all study groups. Exception is made by Group II which recorded, on average, higher concentration values at 30 min assessment than at 15 min. After this span time, the same decreasing tendency was recorded also by Study Group II. We noticed the sudden increase of curcumin concentration in Group II in the sample prelevated at 30 min versus 15 min, followed by a normal decrease, as the one seen in Groups I and III.

All three study groups present a maximum of curcumin quantity, present in rat plasma in the first 40 minutes of the sample assessments. We noticed that the higher quantities correspond to the Group I (treated orally with curcumin, without inflammation). By comparison to literature results, curcumin was found in maximum quantities in the first hour of assessment, which confirms our results.

Also, a study that used administration of curcumin-loaded HPC nanoparticles to rats, showed significant improvement in pharmacokinetic parameters at one hour following the sampling when (is) compared with administration of curcumin suspension [15].

For the statistical analysis of data, the significance of influences was tested and we took into consideration a single influence factor - the group the samples belonged to: Group I, II or III.

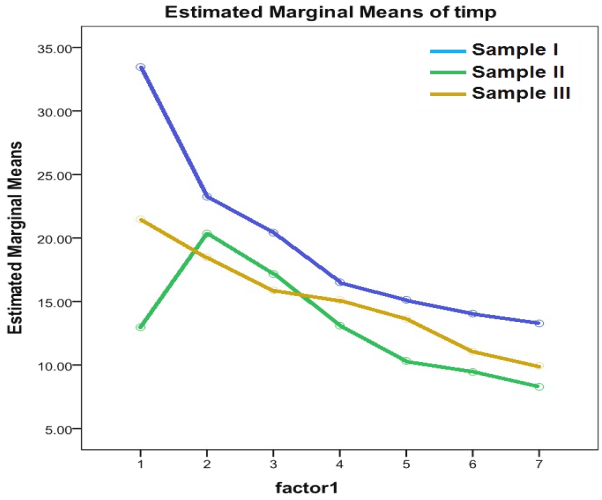


**Figure 3.** Mean curcumin concentration–time profiles in the three study groups. Factor 1 represents the time of sampling for the 7 samples prelevated at 15-30-60-90-120-150-180 minutes.

By taking into account that the constant factor (intercept) as well as the variable factor (the sample) have values lower than 0,05 (even 0.000), we can conclude that both factors contribute significantly to the model. The type of treatment applied influences considerably the variations illustrated by curcumin concentrations.

**Table 1.** Parameters of statistical analysis

| **Tests of Between-Subjects Effects**<br>**Measure: time.**<br>**Transformed Variable: Average** | | | | | |
|---|---|---|---|---|---|
| | **Type III Sum of Squares** | **df** | **Mean Square** | **F** | **Sig.** |
| Intercept | 31741.587 | 1 | 31741.587 | 2658.196 | 0.000 |
| Proba | 886.995 | 2 | 443.498 | 37.141 | 0.000 |
| Error | 179.115 | 15 | 11.941 | | |

We applied the multivariate analysis of variance (MANOVA) and used all the data (all three HPLC determinations for one time frame) not only group mean scores. This analysis certified that the type of the treatment applied to each group, influenced in a significant way the evolution of curcumin HPLC concentration.

The presence of a significant influence was also supported by the 4 multivariate tests (Pillai's Trace, Wilk's Lamba, Hotelling's Trace and Roy Largest Root) included in the table below.

**Table 2.** Multivariate Tests performed on the HPLC results for Groups I-III.

| Effect | | Value | Error df | Sig. |
|---|---|---|---|---|
| factor1 (time) | Pillai's Trace | 0.981 | 10.000 | 0.000 |
| | Wilks' Lambda | 0.019 | 10.000 | 0.000 |
| | Hotelling's Trace | 51.205 | 10.000 | 0.000 |
| | Roy's Largest Root | 51.205 | 10.000 | 0.000 |
| factor1 * Proba | Pillai's Trace | 1.413 | 22.000 | 0.001 |
| | Wilks' Lambda | 0.082 | 20.000 | 0.002 |
| | Hotelling's Trace | 5.184 | 18.000 | 0.005 |
| | Roy's Largest Root | 3.401 | 11.000 | 0.005 |

During the experiment both the singular effect of the passing of time and the compound effect of the passing of time and time of the testing (sampling) were considered. In both cases, all 4 tests registered values of acceptance probability of the nul hypothesis <0.05, which meant that both effects taken into consideration (time and sample) performed a positive influence on curcumin concentration.

For a more precise assessment during the experiment, the estimation of the regression parameters was performed for each separate dependent variable (in our case - each measuring) depending on the analyzed factor. From the statistical analysis point of view, the constant in the model was the mean value of the treatment group (Group III) used as comparison for each measuring.

The coefficients of the other samples showed if, on average, the value of curcumin concentration was higher or lower than that of treatment Group III. Reasonably, it must be interpreted if Sig <0.05.

At 15 min, curcumin concentration recorded in Group I was higher with 11.995 than in Group III. This difference was statistically significant. At 30 min and 60 min, the concentration in Group I was statistically significant and higher with 4.77 and 4.53, than in Group III. On the determination performed at 90 min and 120 min, curcumin concentration in Group I was

higher, with 1.39 and 1.51 than in Group III. By comparing Group I with Group III at 150 min, curcumin concentration in Group I was higher, with 2.98, the difference being statistically significant. With the last measuring with a time frame of 180 min, curcumin concentration in Group I was higher with 3.34 than in Group III, the difference being statistically significant.

In conclusion, in Group I (rat treated only with oral curcumin) the plasmatic concentration of curcumin was higher at any moment of sampling (at 15-30-60-90-120-150-180 min) as compared to Group III (rat with induced inflammation and treated with oral curcumin afterwards). Curcumin concentrations in Group III were lower, probably because of the accelerated metabolism in the presence of inflammation. These values validated the anti-inflammatory effect of the tested substance.

While assessing Group II values at15 minutes we noticed significantly lower curcumin values, with 8.497 than in Group III. Surprisingly, at 30 minutes, and 60 minutes, curcumin concentration in Group II was higher with 1.87 and 1.28 than in Group III. At 90 minutes, curcumin concentration in Group II was lower, with 2.02, than in Group III, and at 120 minutes, curcumin concentration in Group II was lower, with 3.29 than in Group III, the difference being statistically significant. At 150 min and 180 min, curcumin concentration in Group II was lower, with 1.61 and 1.65 on average, than in Group III.

By comparative analysis of Group II (rat to which curcumin was preventively administered and then inflammation was induced) with Group III, we noticed that curcumin concentration was generally lower than in the Group III (rat with induced inflammation and treated with oral curcumin afterwards). In Group II, there were two values higher than in Group III, at 30 min and 60 min respectively, after induced inflammation.These values followed then a descending line, in agreement with the anti-inflammatory effect exercised.

Carboxymethyl cellulose (CMC) is a food additive used as a viscosity thickener and an emulsifier for high concentrations of curcumin in oral administration [22, 28]. A study performed in 2010 on a mouse model proved that both oral curcumin and its CMC emulsifier have anti-inflammatory effects. It also pointed out that curcumin has both preventive and therapeutic effects in the studies with induced murin colitis models [29-32].

Recent curcumin studies described an action of blocking inflammatory pathways, effectively preventing the launch of proteins triggering swelling and pain. Therapeutic effects of curcumin are due to its reduced production of potent proinflammatory mucosal cytokines, as well as the inhibitor effect performed on: phospholipase, lipooxygenase 2, leukotrienes, tromboxane, prostaglandins, nitric oxide, collagenase, elastase, hyaluronidase, monocyte chemoattractant protein-1 (MCP-1), interferon-inducible protein, tumor necrosis factor (TNF) and interleukin 12 (Il-12) [33]. Mercola study showed that the

subjects treated with curcumin had lower levels of blood markers of inflammation such as C-reactive protein (CRP). Phase 1 human trials found no toxicity from curcumin intake and demonstrated the safety of administration [33].

Previous reports from literature on another type of inflammation (colitis) sustain our results based on an acute inflammation model (miositis in rats) underlining the important effect of curcumin in both preventive and therapeutic plans. Even research performed on human subjects concluded that short-term supplementation with bioavailable curcumin significantly improves the inflammatory and oxidative status of patients, suggesting the benefits of preventive administration [34].

Our research protocol did not intend to clarify the nature of curcumin metabolites through the HPLC analysis of plasma. We knew from previous findings that curcumin-glucuronoside, dihydrocurcumin-glucuronoside, tetrahydrocurcumin (THC) and THC-glucuronoside are the major metabolites of curcumin *in vivo* [35].

Several studies showed that orally administered curcumin is absorbed from the alimentary tract and is present in the general blood circulation after being metabolized to the form of glucuronide/sulphate conjugates. Pan study proved that 99% of these curcumin metabolites, in plama were present as glucuronide conjugates [20].

**Oxidative Stress**

Our study aimed to evaluate oxidative stress determination in muscles of the thigh and in organs such as liver and kidney. The samples were collected from the two rats included in the study of Groups I, II and III, as well as from a witness group: two healthy rats who were not exposed to inflammation and did not receive oral Curcumin.

Oxidative stress reflects the level of oxidative damage in a cell, tissue, or organ, caused by the *reactive oxygen species* (ROS), which can be of endogenous sources (energy generation from mitochondria or the detoxification reactions involving the *liver cytochrome P-450 enzyme system*) and exogenous sources: environmental pollutants, alcohol or cigarette exposure, to ionizing radiation, and bacterial, fungal or viral infections [36,37].

Reactive oxygen species, such as free radicals (superoxide and hydroxyl radicals) as well as peroxides (hydrogen peroxide), represent a class of molecules that are derived from oxygen metabolism [38].

Oxidative stress leads to many pathophysiological conditions in the body such as neurological syndromes, gene mutations and cancer, cardiovascular disorders and inflammatory diseases [39,40].

Curcumin has been cited as a natural phenolic compound, with important anti-inflammatory and anti-oxidant properties. It is reported to inhibit production of inflammatory cytokines by peripheral blood monocytes

and alveolar macrophages. It also decreases the inducible nitric oxide synthase (iNOS), cycloxygenase and lipoxygenase and it easily penetrates into the cytoplasm of cells, accumulating in membranous structures such as plasma membrane, endoplasmic reticulum and nuclear envelope [41].

Malondialdehyde (MDA), protein carbonyl (PC) were the oxidizing agents studied in this research. MDA, an important biomarker for oxidative stress, is the end-product of radical-initiated oxidative decomposition of polyunsaturated fatty acids. Its presence is predictive for the occurrence of cardiovascular events or metabolic diseases [42,43].

Proteins are important targets of oxidative changes, too. The changes in the constituent amino acids, caused by oxygen radicals or other activated oxygen species lead to functional or structural protein alterations [44]. The direct oxidation of lysine, arginine, proline and the reactions with MDA produced by lipid peroxidation, determine protein carbonyls to be formed [45].

The oxidation of proteins by reactive oxygen species can be emphasized by determining the PC content [46]. Reactive oxygen species and reactive carbonyl species cause DNA, protein and lipid damage. Collagen structures in the skin are important targets for carbonyl stress, too [45]. Figure 5 presents the results of oxidative stress markers, MDA and PC from blank samples 1 and 2 compared to the sample of Groups I, II, III.

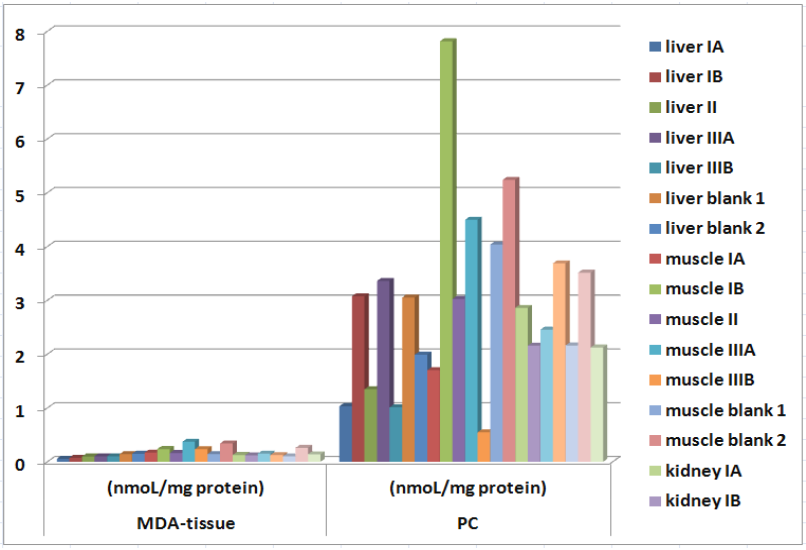


**Figure 5.** Results of the oxidative stress markers (MDA and PC).

For the statistical analysis of the oxidative stress results we took into consideration the small number of observations and we evaluated the normality of MDA distribution and PC. MDA was not normally distributed

(Sig.=0,007) and PC was (Sig =0,064). In both instances, we also performed parametric tests (Student-t) as well as non parametric ones.

When evaluating the 3 types of samples (Groups I to III) ANOVA analysis provided very high values of Sig., which demonstrated that the type of sample did not influence significantly oxidative stress, measured by the variables MDA and PC. Although MDA levels and PC levels assessed from the same sample (muscle, liver or kidney) seemed to be higher in one group, the difference was not statistically significant, probably due to the small number of samples.

**Table 3.** ANOVA analysis of oxidative stress expressed by MDA and PC.

| ANOVA | | | | | | |
|---|---|---|---|---|---|---|
| | | **Sum of Squares** | **df** | **Mean Square** | **F** | **Sig.** |
| MDA | Between Groups | 0.066 | 2 | 0.033 | 8.398 | 0.003 |
| | Within Groups | 0.071 | 18 | 0.004 | | |
| | Total | 0.137 | 20 | | | |
| PC | Between Groups | 10.610 | 2 | 5.305 | 2.206 | 0.139 |
| | Within Groups | 43.283 | 18 | 2.405 | | |
| | Total | 53.893 | 20 | | | |

In the case of MDA, ANOVA provided significant results, indicating that the tissue origin (from muscle, liver or kidney) had a significant influence on the level of oxidative stress. The results were also confirmed by the non-parametric Chi-Square or Kruskal Wallis Test.

Our study results pointed out that oxidative stress was the highest in the muscle.

With reference to PC assessment it can be concluded that there were no significant differences among the tissues examined.

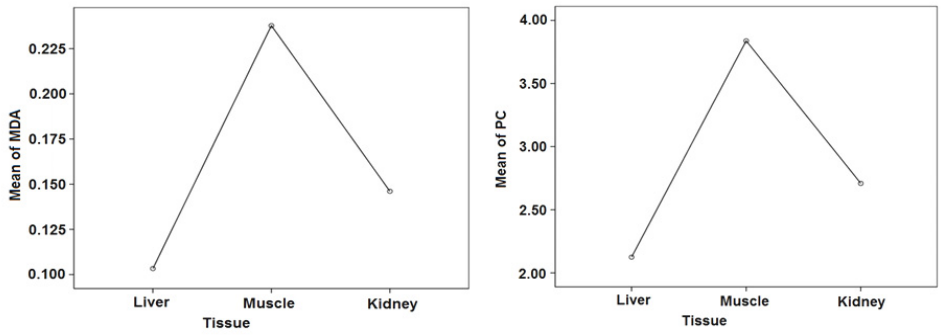


**Figure 6.** MDA and PC results in the three types of tissue

## CONCLUSIONS

Our paper describes a specific and high-performance liquid chromatography (HPLC) method for the determination of curcumin in rat plasma. After protein precipitation performed for each sample, a throughput bioanalysis was done. The assay method was successfully applied to the study of pharmacokinetics of curcumin in the rats included in our study groups. The results obtained by HPLC suggest a decrease of curcumin concentration, with time, in the majority of cases, in all study groups.

The multivariate analysis of variance (MANOVA) certified that the type of the treatment applied to each group, influenced the evolution of curcumin HPLC concentration in a significant way.

Our results based on an acute inflammation model (miositis in rats) underline the important effect of curcumin in both preventive and therapeutic treatment plans. Curcumin concentrations in the study Group III were lower than in Group I, probably because of the accelerated metabolism in the presence of inflammation. The values thus validated the anti-inflammatory effect of the tested substance.

The type of sample from the study groups (Group I-III) did not influence significantly oxidative stress, measured by the variables MDA and PC.

Our study results pointed out that oxidative stress was the highest in the muscle. As far as PC assessment was concerned, it was concluded that there were no significant differences among tissues examined.

## EXPERIMENTAL SECTION

### Instrumentation

The analyses were carried out on a HPLC Jasco Chromatograph (Japan) equipped with an intelligent HPLC pump (Model PU-980), a ternary gradient unit (Model LG-980-02), an intelligent column thermostat (Model CO-2060 Plus), an intelligent UV/VIS detector (Model UV-975) and an injection valve equipped with a 20µL sample loop (Rheodyne). The samples were injected manually with a Hamilton Rheodyne Syringe (50 mL). The system was controlled and the experimental data analyzed were performed with the ChromPass software. Separation was carried out on a Lichrosorb RP-C18 column (25 x 0.46 cm) at 30 ºC column temperature. The mobile phase was a mixture of methanol and 0.1% formic acid (90:10, v/v). The flow rate was 1.2 mL $min^{-1}$ and the UV detection was 425 nm. The mobile phase and samples were filtered through a 0.45 µm membrane.

### Materials and reagents

Curcumin (1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-hepta-diene-3,5-dione, purity > 98%) was purchased from Abcam (BioZyme, Romania). Methanol and formic acid were purchased from Merck (Darmstadt, Germany) and analytical grade water was obtained from Milli-Q Ultrapure water purification system (Millipore, USA). 0.45 µm PTFE filters Syringe were purchased from Nordic Invest (Cluj, Romania). Carboxymethyl cellulose (CMC) was obtained from Abcam (BioZyme, Romania). All reagents used in this study were of analytical grade.

### Standard solutions and spiked samples

Stock solution of curcumin was prepared in methanol at a concentration of 100 mg/mL and stored in amber coloured bottle at 4°C. The curcumin stock solution was diluted with methanol to obtain working solutions of concentration ranging from 1 to 20 µg/mL. All the prepared solutions were protected from light using amber-coloured volumetric flasks. Standard solutions (0.1, 0.2, 0.5, 1, 2 µg/mL) were prepared by spiking 100 µL of each working solution (1, 2, 5, 10 and 20 µg/mL) into 100 µL of blank plasma and 800 µL of methanol. These standards were used to construct calibration curves for the quantification of curcumin in rat plasma concentrations ranging from 100 to 2000 ng/mL.

### Experimental design for drug administration

The experiments were performed on 6 adult female Wistar-Bratislava albino rats (mean age 17 weeks), weighing 200-250 g, bred in the Animal Facility of “Iuliu Hatieganu” University of Medicine and Pharmacy. The animals were housed under controlled conditions (on a 12 hours light-dark cycle, at an average temperature of 22±2°C and ca. 55% relative humidity). They had free access to standard pellets, as basal diet, and water ad libitum, except for 12 hours of fasting before the experiment. 150 mg of Curcumin (Abcam, BioZyme, Romania) was dissolved in carboxymethyl cellulose (CMC) 0.5% and then administered in oral suspension (150 mg curcumin/ kg rat weight). The rats were randomly assigned to three groups (Group I) (n=2). The first group received 0.5 ml / animal oral curcumin suspension (150 mg Curcumin / rat kg body weight) and blood was prelevated at 15, 30, 60, 90, 120, 150 and 180 min. The second group (Group II) was administered curcumin by gavage (preventively) and one hour later terebenthine was injected i.m., followed by blood prelevation at the same time intervals. The rats in the third

group (Group III) received Terebenthine i.m. and after 2 hours curcumin was given by gavage (treatment), then blood prelevation was performed as specified for the other two experimental groups.

After the experiment animals were sacrificed by cervical dislocation. The study protocol was approved by the Institutional Animal Ethical Committee (IAEC) of *Iuliu Hatieganu* University of Medicine and Pharmacy Cluj-Napoca. Experiments were performed in triplicate following the Animal Testing Regulations.

**Plasma sample preparation**

Blood (approx. 1 ml) was collected from the rat retro orbital plexus in an EDTA test tube at 15, 30, 60, 90, 120, 150 and 180 min post dosing of curcumin suspension. Immediately after collection, blood samples were gently inverted several times to ensure complete mixing with the EDTA solutions. The samples were left at room temperature for more than half an hour and then centrifuged at 4500 rpm for 10 minutes. After centrifugation, the upper organic layer was aspirated with a pipette and inserted into a clean Eppendorf tube, resulting a quantity of 0.5 ml plasma (containing three types of proteins: albumins, globulins and fibrinogen) and the curcumin.

For HPLC analysis the rat plasma samples were prepared by using protein precipitation. At each sample of 200 µL rat plasma 800 µL of methanol was added and it was shaken for a few minutes in order to precipitate the plasma protein. The filtrate was then passed through 0.45 µm PTFE filters Syringe and injected into the HPLC system.

**Oxidative stress assessment**

Tissue samples from liver, kidney and muscles were collected from each rat. Biopsy samples helped to determine MDA (nmoL/mg protein), PC levels (nmoL/mg protein) as markers of oxidative stress.

Tissue homogenization was performed using a PT 1200E Polytron homogenizer. The homogeneous medium was obtained using 50 mM Tris-buffered saline + 10 mM EDTA, pH 7.5. A given volume of Tris-buffered saline was added to a tissue sample while stirring in an ice bath. The content was centrifuged for 10 minutes at 1000 x g, 400C, and the supernatant was separated. The total protein concentration of the supernatant was determined using the Bradford method.

MDA was determined by fluorescence lipid peroxidation. Through this process, the resulting MDA reacts with thiobarbituric acid to form a fluorescent adduct. For the determination of tissue homogenate, the sample was boiled for one hour with 10 mM of 2-thiobarbituric solution in 75 mM $K_2HPO_4$, pH 3.

After quenching, the reaction product was extracted with n-butanol. The concentration was measured in the organic phase after its separation by centrifugation. Emission intensity was measured at 534 nm with a Perkin Elmer spectrofluorimeter using the synchronous fluorescence technique to a 14 nm difference between the excitation and emission wavelengths (Δλ). MDA concentration was determined based on a calibration curve consisting of common MDA concentrations using the same measurement technique [47].

The determination of protein carbonyl was carried out by means of a technique which is based on the reaction with a classic carbonyl reagent: 2,4-dinitrophenylhydrazine. The reaction leads to the formation of 2,4-dinitrophenylhydrazone, yellow in color, that can be determined spectrophotometrically. Serum samples were reacted with 10 mM of 2,4-dinitrophenylhydrazine solution in 2.5 N HCl for 1 hour at room temperature in the dark. After being treated with 20% trichloroacetic acid and after the separation of the precipitate obtained by centrifugation, the sample was washed three times with a 1:1 mixture of ethyl acetate and absolute ethanol (v/v). Then, the precipitate was dissolved in 6 M guanidine hydrochloride. From the samples obtained, protein concentration was determined by measuring the extinction at 280 nm. Later, on the same samples, the extinction at 355 nm was also read (wavelength corresponding to the absorp- tion spectra of hydrazones). The protein concentration of the samples analyzed was determined based on a calibration curve consisting of common concentrations of albumin solutions in 6 M guanidine hydrochloride. Simultaneously with the samples treated with 2,4-dinitrophenylhydrazine, blank samples were also processed, only treated with 2.5 N HCl. Extinction was read based on these samples. Carbonyl concentration assessment was done according to the following formula: C = Abs 355 x 45.45 nmoL/mL. Results were expressed as nmol/mg protein, taking into account the protein concentration of the sample expressed in milligrams [46,48].

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