

EXPERIMENTAL STUDY ON THE INFLUENCE OF SULFONAMIDE DRUG RESIDUES FROM HONEY ON BIOCHEMICAL PARAMETERS IN LAB RATS

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Abstract

The objective of the research was the study on the influence of five sulfonamides residues (sulfadiazine, sulfamethazine, sulfathiazole, sulfamethizole, and sulfadimethoxine) in bee honey administered on Wistar rats, by evaluating various biochemical parameters (urea, creatinine, uric acid, total bilirubin, direct bilirubin and transaminases). The biochemical parameters presented significant variations and the results confirmed the occurrence of known toxic effects when administering the five sulfonamides from bee honey in rats: liver dysfunction through the permeabilization of the hepatocyte membrane, and the impairment of hepato-biliary and renal functions.

Rezumat

Scopul cercetărilor a fost studiul influenței reziduurilor a cinci sulfonamide (sulfadiazină, sulfametazină, sulfatiazol, sulfametizol și sulfadimetoxină) în mierea de albine, administrate la șobolani Wistar, prin evaluarea diferiților parametri biochimici (ureea, creatinina, acidul uric, bilirubina totală, bilirubina conjugată și transaminaze). Parametrii biochimici au prezentat variații semnificative și rezultatele au confirmat apariția efectelor toxice cunoscute la administrarea celor cinci sulfonamide în mierea de albine la șobolani: disfuncția hepatică prin permeabilizarea membranei hepatocitelor și afectarea funcțiilor hepato-biliare și renale.

Keywords: sulfonamides, honey, biochemical parameters, rats

Introduction

Sulfonamides are among the most widely used drug substances in veterinary medicine, due to their low price (compared to antibiotics), broad antibacterial spectrum and valuable therapeutic efficacy in some infectious diseases [2, 6, 28, 29]. Antimicrobial chemotherapies are effective against bee diseases, but drug residues may persist for long periods in tissues (sometimes up a month), and the consumption of honey containing such residues represents a potential risk to human health [7, 10, 11, 17, 34].

The intensity, the duration of action and the toxicity of the sulfonamides derive from the body's ability to metabolize those substances [14, 21, 27]. Sulfonamides are considered to have an average toxicity that includes gastrointestinal disorders (nausea, vomiting, diarrhoea), hypersensitivity reactions (allergic) consisting of rash, eosinophilia and rarely anaphylactic shock. Haemolytic anaemia may occur in people with a genetic deficiency of glucose-6-phosphate dehydrogenase. Sulfonamides compete with bilirubin for its binding sites and therefore

should not be administered during the last trimester of pregnancy and to new-borns. Unconjugated bilirubin may be deposited in the basal ganglia of the brain and in the subthalamus, leading to toxic encephalopathy [9, 20, 32].

The toxic effect in humans is explained through the metabolism mechanism of those substances. Sulfonamides are metabolized through Phase I acetylation (Figure 1), but depending on the acetylation capacity of each organism, some part of the absorbed amount is metabolized oxidatively *via* cytochrome CYP450, in lymphocytes and liver microsomes [8, 14, 21]. Excretion of sulfonamides is done in the kidneys by glomerular filtration in unchanged form or metabolites. The elimination of sulfonamides is variable over time, depending on the compound, pharmaceutical form and animal species [3, 16, 18, 26]. At the same time, the proportion of metabolites and the non-transformed substance eliminated differs from species to species [15, 28, 31, 35].

Following rodent biotests, it has been reported that sulfametazine produces tumours with different locations. Evidence of sulfonamide toxicity on the thyroid gland has also been published [12]. In addition, sulfonamides

can sometimes cause drug fever, serum sickness, systemic lupus erythematosus (immunoglobulin G-mediated type III hypersensitivity), and liver toxicity (including necrosis) [7, 12, 15].

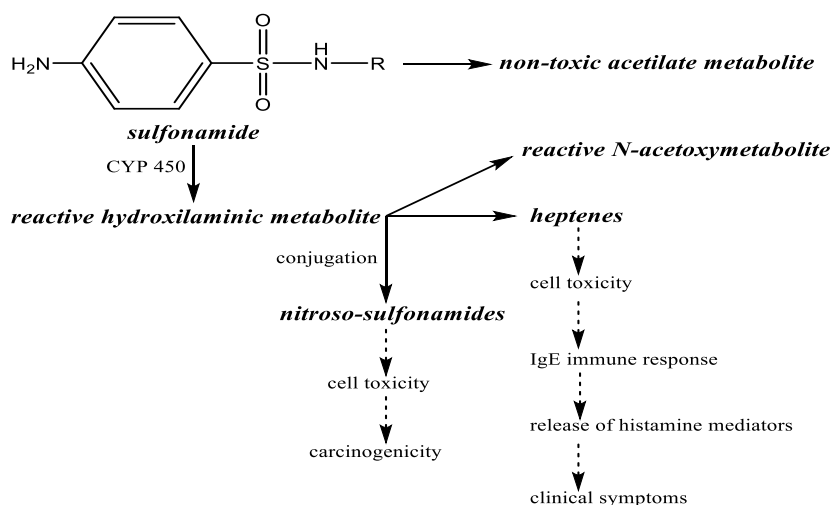


Figure 1.

Metabolism of sulfonamides according to Chițescu & Nicolau (2010)

There is little information regarding the toxicity of sulfonamides residues from honey and its implications on the biochemical parameters [4, 19, 22-25, 33].

The objectives of the research were to study the influence of some sulfonamides residues in honey on Wistar rats, by evaluating various biochemical parameters. The selected sulfonamides are often used by farmers to treat various bee infectious diseases, thus remains of these antibiotics are usually found as traces in bee products.

Materials and Methods

The study was conducted on Wistar female rats weighing 180 - 220 g. The rats were divided into 3 groups: group 1 – the control group, received by gastric gavage standard feed and 2 mL bee honey, group 2 of rats received 2 mL of honey spiked with 100 µg/kg mixture of sulfonamide by gastric gavage for 5 days and group 3 received 2 mL of honey spiked with 100 µg/kg mixture of sulfonamide by gastric gavage, for 14 consecutive days. The mixture of sulfonamides (Figure 2) contained 20 µg of each substance (sulfadiazine, sulfamethazine, sulfathiazole and sulfamethizole, sulfadimethoxine).

Blood samples were collected in vacutainers containing anticoagulant (1 part 1% EDTA-Na₂ anticoagulant to 9 parts of whole blood), and they were used immediately to determine the biochemical parameters. The study was conducted in accordance with the approval issued by the Research Ethics Committee of the “Grigore T. Popa” University of Medicine and Pharmacy Iași, Romania.

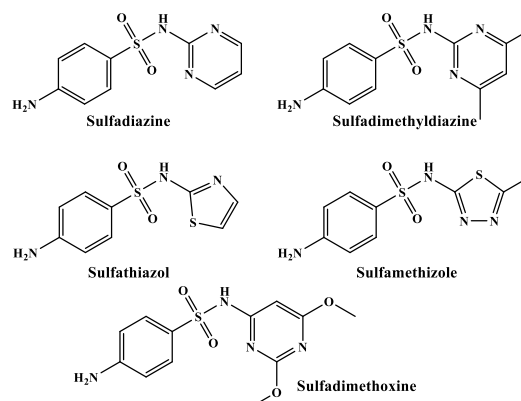


Figure 2.

Chemical structure of the sulfonamides included in the study

The quantitative determinations of the biochemical parameters (urea, creatinine, uric acid, total bilirubin, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT)) were performed on the Rx Imola automatic analyser produced by Randox Laboratories, UK.

The quantitative determination of urea was performed based on the UV enzymatic kinetic method with urease. Briefly, urea was hydrolysed by urease to ammonium ion and CO₂. The ammonia produced was combined with α-ketoglutarate and NADH in the presence of glutamate dehydrogenase, when glutamate and NAD⁺ were obtained. The change in absorbance due to the formation of NAD⁺ instead of the consumed NADH was proportional to urea concentration [30].

The quantitative determination of creatinine was based on its reaction with picric acid in alkaline medium.

The concentration of the newly formed compound was directly proportional to creatinine concentration. For the quantitative determination, the uric acid was converted by uricase to allantoin and hydrogen peroxide. The latter, finally formed quinonimine with 4-aminophenazone, whose colour was directly proportional to uric acid concentration.

Quantitative determination of total bilirubin was performed based on the Jendrassik Grog colorimetric method [5]. Total bilirubin was released from the albumin molecule in the presence of caffeine and reacts with diazotized sulfanilic acid to form a coloured compound whose colour intensity was directly proportional to total bilirubin. Determinations on haemolysed samples were avoided because it interfered with the analysis.

The determination of direct (conjugated) bilirubin was based on the ability of direct bilirubin to react with sulfanilic acid to form an intermediate reaction compound to which the diazo reagent was added. A red compound was formed, and colour intensity was directly proportional to serum concentration of direct bilirubin.

Quantitative determinations of ALT and AST respectively, were performed using the standardized UV method,

according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). The decrease in absorbance measured at 340 nm, due to NADH consumption and NAD⁺ formation, was inversely proportional to the activity of the enzyme.

Statistical analysis

The data obtained were statistically evaluated in order to eliminate the biological variations and the determination errors. The statistical description of the samples was done in order to obtain the descriptors of interest (mean, median, standard error of mean (SE), standard deviation, amplitude, Skewness coefficient, Kurtosis coefficient). Also the Kolmogorov-Smirnov test was applied to assess the normality of the data distribution. The values were considered as following: p < 0.05 – significant, p ≤ 0.01 – distinctly significant, p ≤ 0.001 – highly significant.

Results and Discussion

The processing of the data obtained in determining the biochemical parameters, by applying descriptive statistical tests and significance, led to the results presented in Tables I and II.

Table I

Experimental data for the group of rats that received a 5-day treatment with sulfonamides spiked honey

| Statistical parameter | Creatinine (mg/dL) | Urea (mg/dL) | Uric Acid (mg/dL) | Total bilirubin (mg/dL) | Direct bilirubin (mg/dL) | AST (IU/L) | ALT (IU/L) |
|---------------------------|--------------------|---------------|-------------------|-------------------------|--------------------------|---------------|---------------|
| Mean ± SE | 0.45 ± 0.02 | 17.04 ± 1.70 | 1.71 ± 0.13 | 0.62 ± 0.02 | 0.08 ± 0.01 | 60.71 ± 3.87 | 72.75 ± 1.85 |
| Median | 0.47 | 17.00 | 1.64 | 0.63 | 0.07 | 61.20 | 74.64 |
| Standard deviation | 0.05 | 3.80 | 0.29 | 0.04 | 0.02 | 8.66 | 4.14 |
| Variance | 0.002 | 14.47 | 0.08 | 0.001 | 0.0003 | 75.00 | 17.14 |
| Skewness coefficient | -0.53 | 0.98 | 0.68 | -0.58 | -0.32 | -0.10 | -0.53 |
| Kurtosis coefficient | -3.04 | 1.43 | 0.98 | -1.22 | -0.74 | -1.67 | -3.01 |
| Amplitude | 0.1 | 10.10 | 0.94 | 0.10 | 0.04 | 21.31 | 8.64 |
| Minimum value | 0.39 | 12.90 | 1.36 | 0.56 | 0.05 | 49.82 | 67.79 |
| Maximum value | 0.49 | 23 | 2.14 | 0.66 | 0.10 | 71.13 | 76.43 |
| Confidence level (95%) | 0.06 | 4.72 | 0.39 | 0.05 | 0.02 | 10.75 | 5.14 |
| Confidence interval (95%) | 0.39 - 0.50 | 12.32 - 21.76 | 1.35 - 2.07 | 0.57 - 0.67 | 0.05 - 0.10 | 49.95 - 71.46 | 67.61 - 77.89 |

Table II

Experimental data for the group of rats that received a 14-day treatment with sulfonamides spiked honey

| Statistical parameter | Creatinine (mg/dL) | Urea (mg/dL) | Uric Acid (mg/dL) | Total bilirubin (mg/dL) | Direct bilirubin (mg/dL) | AST (IU/L) | ALT (IU/L) |
|---------------------------|--------------------|---------------|-------------------|-------------------------|--------------------------|-----------------|-----------------|
| Mean ± SE | 0.72 ± 0.02 | 18.36 ± 0.39 | 2.40 ± 0.19 | 1.27 ± 0.04 | 0.32 ± 0.03 | 109.57 ± 3.29 | 128.23 ± 2.68 |
| Median | 0.74 | 18.60 | 2.28 | 1.22 | 0.35 | 110.18 | 130.61 |
| Standard deviation | 0.03 | 0.87 | 0.42 | 0.09 | 0.07 | 7.36 | 5.99 |
| Variance | 0.001 | 0.76 | 0.18 | 0.008 | 0.005 | 54.21 | 35.90 |
| Skewness coefficient | -0.59 | -1.09 | 0.32 | 1.09 | -1.02 | 0.45 | -0.99 |
| Kurtosis coefficient | -2.98 | 0.53 | -3.04 | 0.06 | -0.30 | -0.33 | 0.66 |
| Amplitude | 0.07 | 2.15 | 0.87 | 0.23 | 0.17 | 18.19 | 15.51 |
| Minimum value | 0.68 | 17.00 | 1.98 | 1.18 | 0.21 | 101.17 | 119.00 |
| Maximum value | 0.75 | 19.15 | 2.85 | 1.41 | 0.38 | 120.08 | 134.51 |
| Confidence level (95%) | 0.04 | 1.08 | 0.52 | 0.12 | 0.09 | 9.14 | 5.00 |
| Confidence interval (95%) | 0.68 - 0.76 | 17.27 - 19.44 | 1.87 - 2.92 | 1.15 - 1.38 | 0.23 - 0.40 | 110.43 - 118.71 | 120.79 - 135.67 |

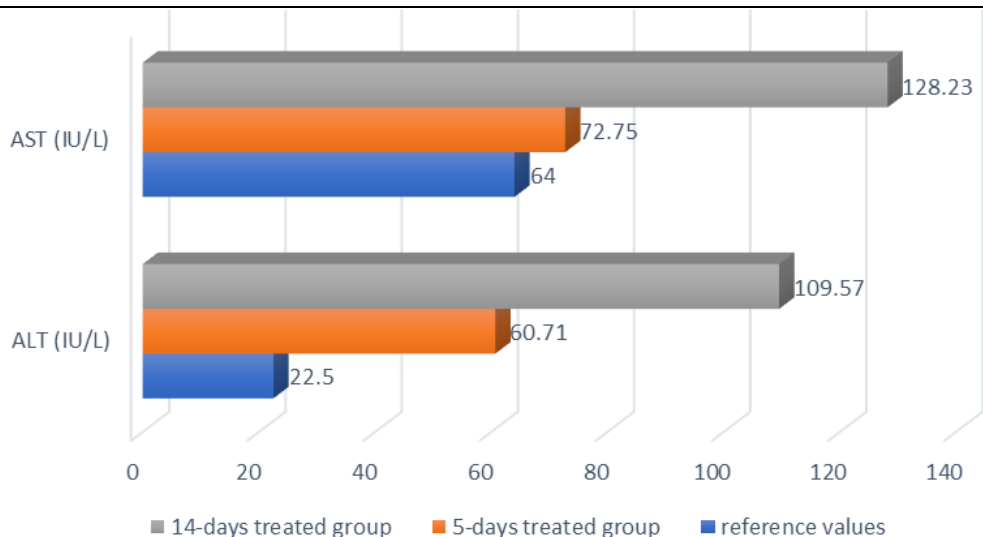


Figure 3.

Changes of transaminases levels after 5 days and 14 days of treatment with spiked honey

As expected, the consumption of contaminated honey for two weeks led to significantly elevated levels of ALT and AST as compared to 5 days treatment (Figure 3).

Serum concentrations of AST and ALT increased following administration of honey with sulfonamides, compared with the values determined in the control group. Those results were in agreement with data from the literature, according to which the toxic effect of sulfonamides, even administered in very small quantities, manifested by modifying the liver parameters in

laboratory animals [1]. Thus, ALT and AST activity recorded statistically significant increases ($p < 0.0001$) in rats that received honey spiked with sulfonamides, compared to the control group, thus proving the change in hepatocyte membrane permeability and the degree of liver dysfunction in rats.

However, more important changes were observed on the biliary pathways (Figure 4). Similar effects were observed on zebrafish metabolomic model from sulfonamides contaminated water [9].

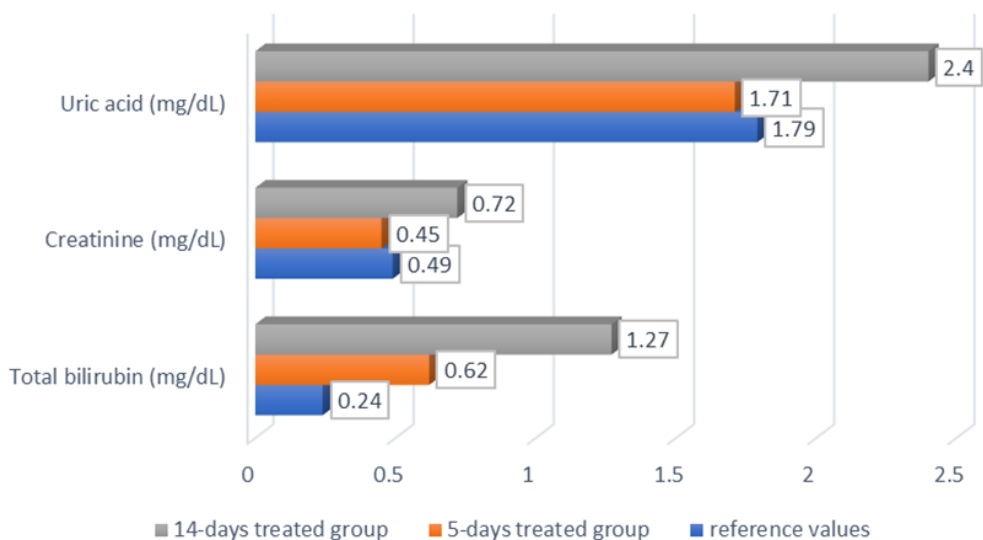


Figure 4.

Comparison between the experimental data and the reference values for female Wistar rats

As the groups were small ($n \leq 15$), the Kolmogorov-Smirnov test was applied to assess the normality of the data distribution, as well as for the other parameters analysed in that part of the study. The application of the statistical test highlights the existence of a significant difference According to the evaluated

parameters, for both treated groups we established that the samples had both normal and similar distribution, so they were comparable. Moreover, two tailed t-Student indicated that the obtained results were very significant ($p < 0.001$, $T = 2.92$, $r = 0.9871$, $R^2 = 0.7068$, $t = 1.86$) for all tested parameters except for urea

and uric acid, when the significance was distinctive with $p < 0.015$ and $p < 0.01$ respectively. Therefore, all the obtained values were statistically significant in terms of changes from the control values.

Noteworthy is that Romanian bee honey samples are not the only contaminated products, residues of veterinary medicines (chloramphenicol, sulfathiazole and tetracycline) were found in Spanish, Belgian, Turkish and Chinese market samples [2, 4, 6, 14, 21, 22, 25, 27, 29]. Therefore, precise methods are important for proper detection of contaminants [14, 18, 19, 26, 31, 33, 34]. However, the true impact of the consumption of contaminated honey was not assessed on animal models thus far. To date, only one study has tested the relevance of sulfonamides metabolites found in soil and water on zebrafish [9]. Our research showed a statistically insignificant increase in uraemia (18.36 ± 0.87 mg/dL) for the sulfonamide-treated groups, compared with the reference group for which the average urea concentration was 17.04 ± 3.80 mg/dL.

Serum values of urea are dependent on three factors: protein catabolism, diuresis and renal functional capacity, thus it was useful to interpret the results for that parameter by correlating it with the results of other determinations such as creatinine and uric acid.

The effect of the administration of honey with sulfonamide in rats on renal function resulted in a significant increase in the values of uric acid and creatinine concentrations ($p \leq 0.05$) in the test groups compared with the control group.

Creatinine synthesis occurs in the liver and kidneys. Creatinine, like urea, is excreted in urine. Both substances are found in constant physiological concentrations in the blood, which transport them. Creatinine concentration in urine can be used as a reference value for excretion of albumin or α -amylase [1, 2, 10, 11].

Evaluation of liver activity by analysing total bilirubin and direct bilirubin revealed a significant increase in total bilirubin concentration ($p < 0.0001$) following administration of bee honey spiked with sulfonamide. The average concentration obtained on the tested group was 1.27 mg/dL (14 days treatment), compared to the shorter treatment group (5 days) with an average concentration of 0.62 mg/dL. The values of the direct bilirubin concentration varied similarly to those of total bilirubin. In the group that consumed honey spiked with sulfonamides for 14 days, the values of the direct bilirubin were 0.32 ± 0.07 mg/dL compared to 0.08 ± 0.017 mg/dL recorded for the control group. Similarly, the Korean researchers concluded that xenobiotics and its metabolites influence the hepatobiliary metabolic pathways [9].

Other research groups found that sulfonamides, especially nitrogen including sulfonamides and its metabolites, have antigenic potential on T-cells inducing allergy [7, 10, 11]. From our data, we can conclude that the presence of sulfonamides in honey and bee products

has a negative impact on liver and renal status. Therefore, Romanian legislation should follow the provisions of countries like Germany, Switzerland and Japan which forbade the use of such antibiotics for the treatment of bees [2, 6, 10, 14].

Conclusions

The biochemical parameters were modified significantly following the administration of bee honey spiked with sulfonamides to Wistar rats. The evolution of ALT and AST values showed significant increases in their activities, suggesting the permeabilization of the hepatocyte membrane and the migration of both enzymes into the intercellular space. That permeabilization of the hepatocyte membrane might have been due to the consequent damage caused by the binding of the sulfonamides or their metabolites to the membrane proteins.

The increase of plasma concentrations of total bilirubin and direct bilirubin confirmed the severe impairment of hepato-biliary function following the administration of honey containing sulfonamides by gastric gavage for 14 days.

Moreover, the parameters of the investigation of the renal function recorded increased concentrations in the tested groups, due to the toxicity of honey containing sulfonamides. Therefore the results confirm the toxic potential of sulfonamides even in low doses or as traces in foods. Both beekeepers and consumers should be advised to avoid the consumption of such products.

Conflict of interest

The authors declare no conflict of interest.

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