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TISSUE DISTRIBUTION, EXCRETION AND METABOLISM OF O-ANISIDINE IN RATS

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Abstract
Objectives: The principal commercial use of o-anisidine is believed to be as an intermediate in the manufacture of dyes. It has also been reported to be an intermediate in the manufacture of synthetic guaiacol and its derivatives. o-Anisidine is an urinary bladder carcinogen in mice and rats. The aim of the study was to investigate the kinetics of body distribution, excretion and biotransformation of o-anisidine in rats following a single, intraperitoneal administration. **Materials and Methods:** The tissue distribution and excretion of o-anisidine following i.p. administration of a single dose of 10 mg/kg was investigated using radiotracer [^{3}H]. Metabolism of o-anisidine was investigated in the rats following i.p. administration of a single dose of 50 mg/kg using GC/MS technique. **Results:** After 72 h, about 72% of the given dose was excreted in urine. As indicated, urine proved to be the main route of tritium excretion. In all examined tissues, the highest concentrations of tritium were found 12 h after injection and the highest accumulation was detected in the liver, kidneys and in the muscle tissue. In urine, the following substances were identified and quantified by GC peak areas: N-acetyl-2-methoxyaniline and N-acetyl-4-hydroxy-2-methoxyaniline. **Conclusions:** Prolonged tritium retention observed in the majority of tissues indicated that o-anisidine, especially in the case of repeated exposure, might accumulate in the body. The metabolism encompasses amine group acetylation and ring oxidation.

Key words:
o-Anisidine-ring-U-^{3}H, Distribution, Excretion, Metabolism, Rats

INTRODUCTION

The principal commercial use of o-anisidine is believed to be as an intermediate in the manufacture of dyes. It has also been reported to be an intermediate in the manufacture of synthetic guaiacol and its derivatives [1,2]. The oral LD_{50} of o-anisidine has been reported to be 2000 mg/kg b.w. in rats, 1400 mg/kg b.w. in mice and 870 mg/kg b.w. in rabbits. Subacute effects include hematological changes, anemia and nephrotoxicity [2]. o-Anisidine hydrochloride was tested in mice and rats by dietary administration. It was carcinogenic in both species, producing transitional-cell carcinomas of the urinary bladder and was also mu-

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tagenic and genotoxic [3–7]. So far *in vivo* metabolism of o-anisidine has not been investigated. Some *in vitro* metabolism investigations with use of horseradish peroxidase resulted in one-electron oxidation to free radicals. The above enzyme oxidized o-anisidine to a diimine metabolite, which subsequently hydrolyzed to form a quinone imine [8,9].

We, therefore, decided to investigate the metabolism of o-anisidine in rats. The aim of the present study was to investigate the kinetics of body distribution, excretion and biotransformation of o-anisidine in rats following a single, intraperitoneal administration.

MATERIALS AND METHODS

Chemicals

o-Anisidine-ring-U-^{3}H (AN-^{3}H), with a specific activity of 336 MBq/g, chromatographically pure, was obtained from the Institute of Radiation, Faculty of Chemistry, Technical University of Łódź, Poland. Unlabelled (cold) o-anisidine was purchased from Aldrich (England). All the other chemicals (Aldrich) were of analytical grade.

Animals

Adult male outbred IMP: WIST (Rattus) rats of 200–220 g b.w., were obtained from the breeding colony of the Nofer Institute of Occupational Medicine in Łódź. The animals were supplied at least one week before the experiment and were fed a standard palletized diet Murigram (Agropol, Motycz, Poland) and had free access to water.

Animal treatment

The animals were put individually in glass metabolism cages (Simax, the former Czechoslovakia) and allowed to acclimatize for 2 days (48 h). Subsequently, rats (groups n = 6 or n = 8) were administered intraperitoneally, a single dose of 10 mg/kg o-anisidine-ring-U-^{3}H (as free base) dissolved in olive oil (about 1655 kBq per animal).

Immediately after administration, the rats were placed individually into glass metabolism cages, which enabled the collection of separate samples of urine and feces.

Sampling of biological material and measurements of ^{3}H-radioactivity

Blood samples were collected (0–72 h) from the tail veins of 8 rats using calibrated, heparinized capillaries following a single administration of the compounds (1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 48 and 72 h after administration); 0.03 ml of blood was collected each time. Rats were decapitated under light-ether narcosis at appropriate time intervals and examined tissues were removed for determination of radioactivity. In all the experiments the Polish law on animals protection was followed [10].

The kinetics of tritium activity in blood was carried out using Sigma Plot 3.0 (Jandel Corporation) for Windows. Tissue homogenates (20%), feces water homogenates (10%) and erythrocytes were digested according to the method of Mahin and Lofberg [11]. Urine samples, diluted with water to 50 ml, and plasma samples were measured directly.

All radioactivity measurements were carried out using Racbeta 1209 (LKB, Sweden), liquid scintillation counter and Hydroluma from Baker (Germany) as the scintillation mixture. Counting correction was achieved using the external standard method.

Isolation and identification of urinary metabolites

Six rats were i.p. given a single dose of 50 mg/kg o-anisidine (cold) dissolved in olive oil. Immediately after administration, the rats were placed individually into glass metabolism cages, which enabled the collection of separate samples of urine and feces.

Samples of urine (5 ml) were collected during the first 24h. In order to secure a sufficient yield in extraction of alkaline metabolites, samples were alkalized with sodium bicarbonate (pH9) at room temperature and extracted twice with 10 ml of diethyl ether. Samples of physiological urine of rats (5 ml) were also extracted at the same pH. The ether extracts were evaporated to near dryness, dissolved again in a small amount of ether (about 1 ml) and 1 μl was injected into a GC/MS system. The mass detector was operated in SCAN mode at a mass range of 15–350 U. The GC/MS system from Hewlett Packard consisted of a 5970 MSD gas chromatograph and ChemStation 59970C. Working parameters were as follows: the

injector temperature was 250°C; the oven was programed for an initial temperature of 50°C/min (2 min), immediately followed by a linear rise of 5°C/min (10 min), then followed by a temperature of 100°C for 5 min and a linear rise of 10°C/min (15 min). The terminal oven temperature was 250°C for 8 min. Helium was used as the carrier gas.

The identification of metabolites was based on comparisons of the sample mass spectra with mass spectra contained in Wiley's computer database. In the absence of the latter, mass spectrum analysis was performed. The amounts (expressed in percent) of the identified metabolites were roughly calculated on the basis of comparisons of the peak areas presented in a chromatographic diagram (total area of all substances being identified 100%).

RESULTS

The excretion of tritium after a single, i.p. administration of o-anisidine-ring-U-^{3}H (AN-^{3}H) at a dose of 10 mg/kg is presented in Table 1.

As indicated, urine proved to be the main route of ^{3}H excretion. Almost 72% of the given compound was excreted during the first 72 h, and about 6% with feces.

The kinetics of radioactivity decline in blood plasma during 72 h after a single, i.p. administration of AN-^{3}H is presented in Fig. 1. Decline of ^{3}H in plasma was biphasic. The half-lives for fast and slow phases were about 1.5 h and 80 h, respectively. The kinetics of radioactivity decline in erythrocytes is presented in Fig. 2. Decline of ^{3}H in erythrocytes was also biphasic. The half-lives for fast and slow phases were about 1.0 and 116 h, respectively. The tissue

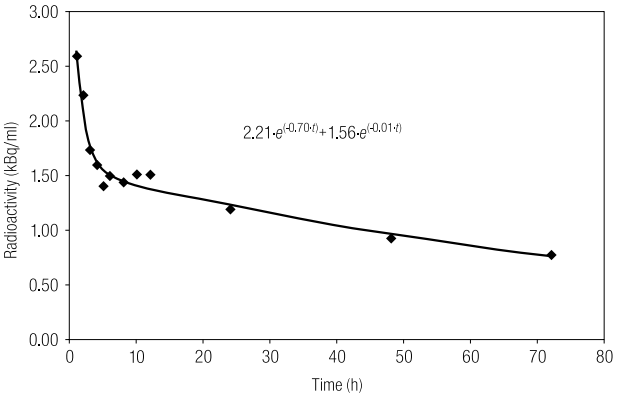


Fig. 1. Kinetics of ^{3}H in plasma following a single, i.p. administration of o-anisidine-ring-U-^{3}H at a dose of 10 mg/kg b.w. (1650 kBq per rat) in rats. Results are means from eight rats. SD ≤ 20%.

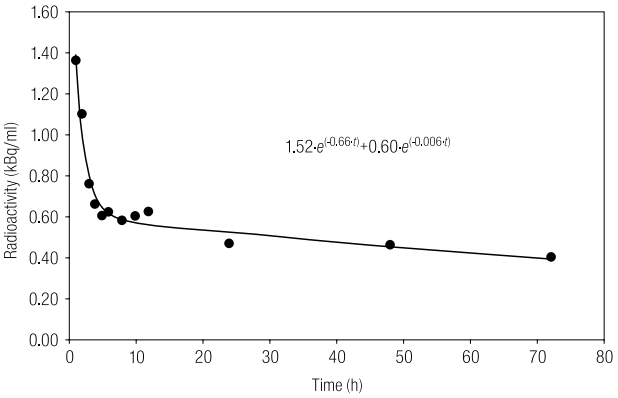


Fig. 2. Kinetics of ^{3}H in erythrocytes following a single, i.p. administration of o-anisidine-ring-U-^{3}H at a dose of 10 mg/kg b.w. (1650 kBqper rat) in rats. Results are means from eight rats. SD ≤ 20%.

and organ distribution of tritium after a single, i.p. administration of AN-^{3}H is shown in Fig. 3. In all examined tissues, the highest levels of tritium accumulation were found 12 h after injection, except for kidneys and fat tissues. The highest levels of tritium were found in the liver, kidneys, and in the muscle tissue. In most examined tissues a moderate

Table 1. Total balance of tritium following a single, i.p. administration of o-anisidine-ring-U-^{3}H

Medium	Percentage of administered dose ($\bar{x}$ ± SD), n = 6			
	0–24 h	24–48 h	48–72 h	0–72 h
Urine	54.60 ± 5.05	11.87 ± 1.65	5.38 ± 0.84	71.49 ± 3.07
Feces	3.72 ± 1.00	1.77 ± 0.61	0.90 ± 0.21	6.15 ± 1.70
Blood	1.76 ± 0.12	1.22 ± 0.06	1.12 ± 0.08	1.12 ± 0.08
Muscles	8.54 ± 0.64	8.30 ± 0.51	7.79 ± 0.77	7.79 ± 0.77
Remaining tissues	2.87 ± 0.02	1.77 ± 0.02	1.64 ± 0.01	1.64 ± 0.01
Total	71.49	24.92	16.84	88.55

Explanation: all results are means from six rats ± SD; to assess ^{3}H distribution, blood was accepted as 7ml per 100 g b.w.; fat tissue, 12% and muscles, 40% of the whole body weight [12,13].

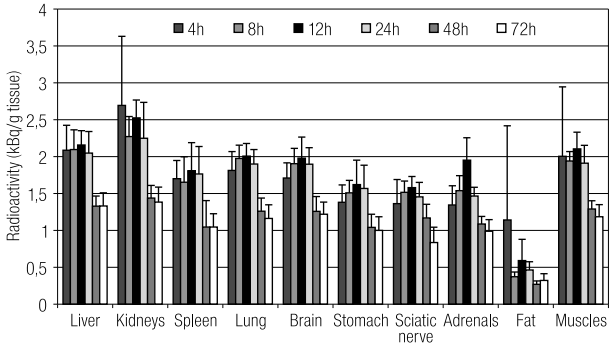


Fig. 3. The specific activity of ^{3}H in the tissue after i.p. administration of o-anisidine at a dose of 10 mg/kg. Results are means from six rats ± SD.

decline in tritium levels with time was observed. The total amount of tritium accumulated in tissues, together with the amounts excreted is presented in Table 1. After 72 h, urine and feces played a decisive role in the total balance.

Identification of o-anisidine metabolites

The yield of ether extraction of o-anisidine metabolites from urine at pH = 9 based on radioactivity recovery, was about 75 ± 10%. Chromatograms of the urine extracts obtained after exposure of o-anisidine were compared with those for control urine samples. In selecting peaks for further analysis, two criteria were used: (a) the absence of the peak in control urine, and (b) the presence of aromatic structure.

Gas chromatogram of ether extracts of urine indicated the presence of 3 peaks potentially related to the administration of o-anisidine (Fig. 4). The peaks were further analyzed by mass spectrometry, as described in the Methods section.

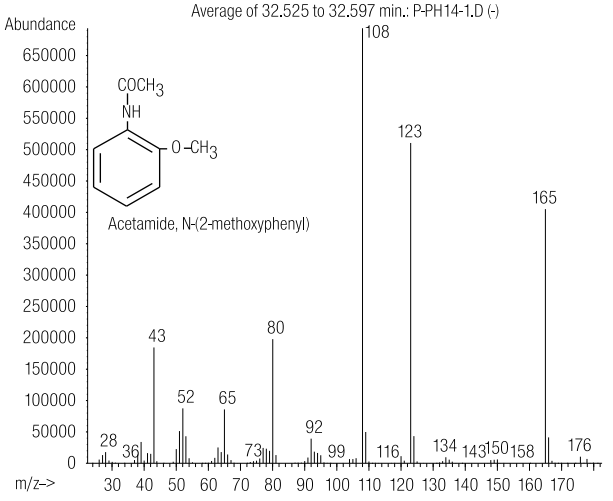


Fig. 5. A typical mass spectra of the examined substances (main peak).

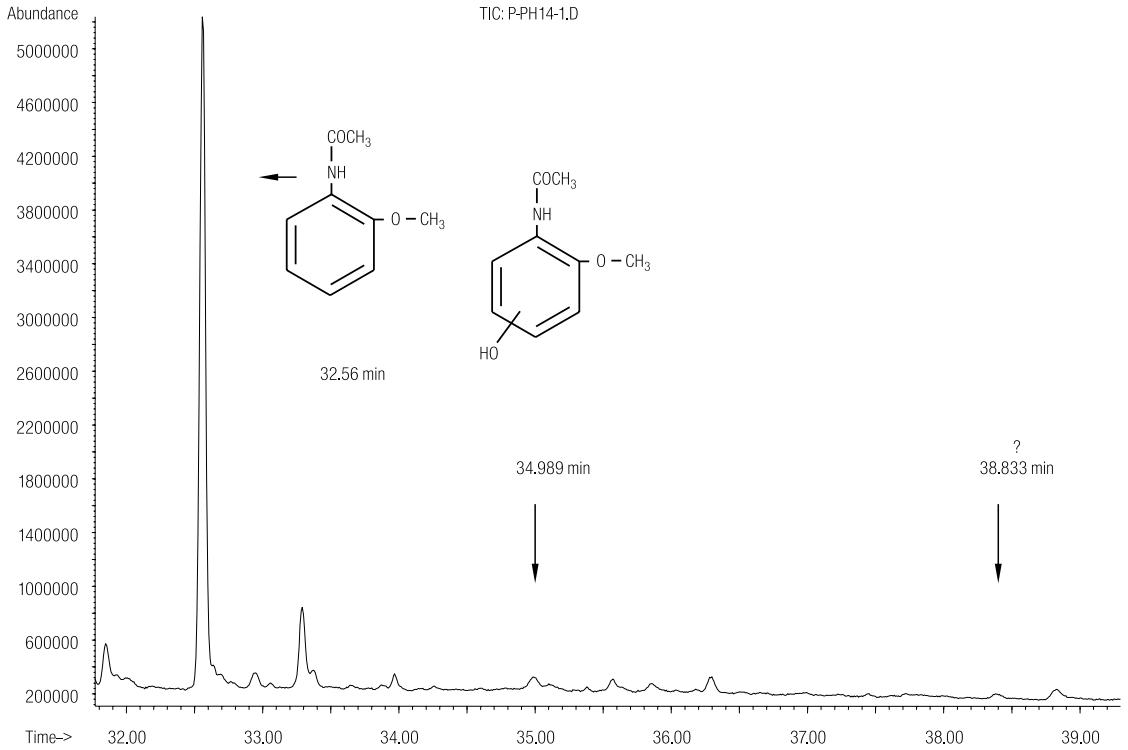


Fig. 4. A typical gas chromatogram of the ether extract of urine following administration of o-anisidine.

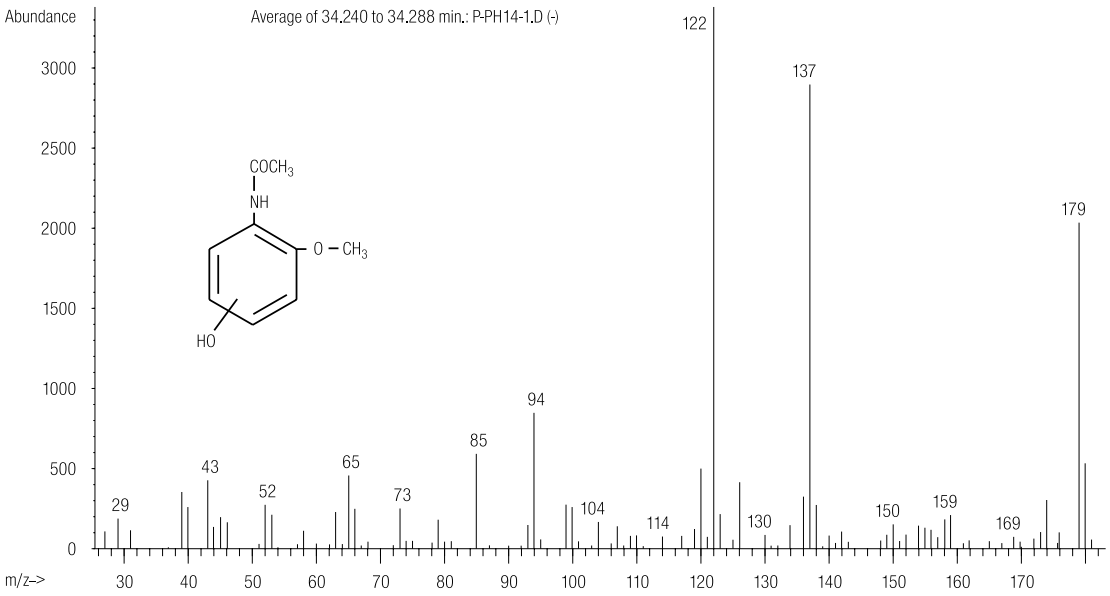


Fig. 6. A typical mass spectra of the examined substances present in the peak with retention time of 34.99 min.

Two urinary substances, shown in Fig. 4, were identified: (1) N-acethyl-2-methoxyaniline and (2) N-acetyl-4-hydroxy-2-methoxyaniline. We were not able to identify a substance, present in a third peak, which had a retention time of 38.83 min.

An example of typical mass spectra analysis is shown in Figs. 5 and 6. Based on the comparison of the peak surfaces in the chromatographic diagram (Fig. 4), the relative contribution of individual metabolites was calculated (total surface = 100%). The main urinary metabolite resulting from the administration of o-anisidine were (1) N-acetyl-2-methoxyaniline (almost 97% of the total amount excreted with urine) and (2) N-acetyl-4-hydroxy-2-methoxyaniline (about 1.5% of the total amount excreted with urine).

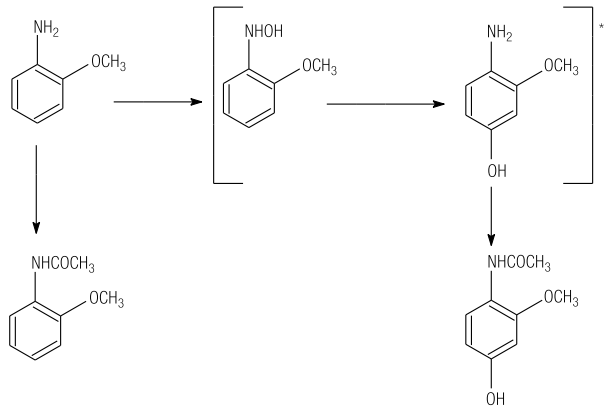
DISCUSSION

The disposition and metabolism of o-anisidine have not been studied in mammals. This report presents data on the distribution and excretion of o-anisidine obtained by radiotracer studies in rats as well as on the identification of the main metabolites using GC/MS technique.

It results from the present study that o-anisidine administered i.p. once is quickly absorbed into blood plasma of the rat and is further distributed into organs (tissues). The rats were administered o-anisidine tritiated in aromatic ring, which enabled us to observe the whole molecule of the investigated compound. However, there was a certain limitation of the method, the marker was measured without knowing chemical structures. The radioactivity level was comparable in all the investigated tissues, except fatty tissues, where it was significantly lower, whicht was probably associated with low lipophilicity of the investigated compound. Having reached the maximal concentration (12 h after administration), a slow decrease in radioactivity was observed at subsequent time points with urine as the main route of excretion. Approximately, within 72 h, 71% of the administrated dose was excreted in urine.

Following calculations of the balance of total tritium excreted and stored, it was found that o-anisidine belongs to compounds of moderate turnover rate in the rat body. Prolonged tritium retention observed in the majority of tissues points to a possibile of accumulation of this compound in case of repeated exposure.

Quantitative calculations of tritium deposited in tissues should be treated as approximate values due to the fact that they are burdened with some error, resulting from theoretical assumptions on the percentage share of muscular and fatty tissues with relation to the total mass of the rat. Tritium radioactivity was measured in selected tissues and organs, and thus it was not measured in the alimentary tract and its contents, skin, bones or tendons.



* Tentative metabolites analogous to biotransformation of aniline.

Fig. 7. Tentative metabolic pathways of o-anisidine in rats.

Identification of main metabolites of o-anisidine in the rats urine demonstrated the presence of two major products of metabolism: N-acetyl-2-methoxyaniline and N-acetyl-4-hydroxy-2-methoxyaniline. In the literature, there is a lack of *in vivo* studies of o-anisidine metabolism. Whereas, in *in vitro* studies with horseradish peroxidase, o-anisidine was oxidized to diimine metabolite, which subsequently hydrolyzed to form quinone imine. The quinone imine formed a conjugate with glutathione and was also reduced by glutathione or ascorbic acid. Using tritium-labeled anisidine, the authors observed substantial metabolism-dependent covalent biding of both isomers to protein and DNA [8,9]. In our study, these metabolites were not detected in the analyzed urine.

Based on metabolites first identified in this study, and on the literature data, o-anisidine metabolism pathway is suggested in the rat (Fig. 7). Quantitatively, the main metabolism pathway leads through amine group acetylation (conjugation) to N-acetyl-2-methoxyaniline.

Acetylation is the most frequent conjugation reaction of many aromatic amines. It may be acknowledged that in the above mentioned *in vitro* studies, acetylation of the amine group in the experimental conditions did not take place, which enabled efficient oxidation of the free amine group to the final quinone imine form. Thus the differences in biotransformation of o-anisidine are observed *in vivo* conditions, in which conjugation is not limited by deficiency of substrates, contrary to *in vitro* conditions. It is most likely that the presence of both groups (methoxyl and amine) of the o-anisidine molecule "close to each other" essentially affects biotransformation of this compound towards conjugation.

Formation of another identified metabolite containing hydroxyl group in aromatic ring may proceed similarly to biotransformation of aniline, whose main metabolite, p-aminophenol, is excreted with urine. Then a similarly formed 4-hydroxy-2-methoxyaniline undergoes N-acetylation and conjugates probably (on hydroxyl group) with sulphuric and/or glucuronic acid. However, this fragment of o-anisidine pathway of biotransformation requires further research.

REFERENCES

1. Arner RC, Nutman CA. *Testing of some azo dyes and thier reduction products for mutagenicity using Salmonella typhimurium TA1538*. Mutation Res 1977; 44: 9–19.
2. *Monographs on the Evaluation of the Carcinogenic Risk of Chemical to Humans.* Vol. 27. Lyon: International Agency for Research on Cancer; 1982. p. 63–77.
3. Haworth ST, Lawlor K, Mortelmans W, Speck W, Zeiger E. *Salmonella mutagenicity test results for 250 chemicals.* Environ Mutagen 1983; 5 Suppl. 1: 1–142.
4. Dunkel VC, Zeiger E, Brusick D, McCoy E, Mac Gregor D, Mortelmans K, et al. *Reproducibility of micobial mutagenicity assays: 11. Testing of carcinogenes and noncarcinogenes in Salmonella typhimurium and Enscherichia Coli.* Environ Mutagen 1985; 7 Suppl. 5: 1–248.
5. Galoway SM, Armstrong MJ, Ruben C, Colman S, Brown B, Cannon C, et al. *Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells, evaluations of 108 chemicals.* Environ Mol Mutagen 1987; 10 Suppl. 10: 1–175.
6. Garberg P, Aakerblom EL, Bolcsfoldi G. *Evaluation of a genotoxicity test measuring DNA-strand breaks in mouse lymphoma cells by alkaline unwinding and hydroxyapatite eluation.* Mutation Res 1988; 203: 155–76.
7. Thompson DC, Josephy PD, Chu JW, Eling TE. *Enhanced mutagenicity of anisidine isomers in bacterial strains containig elevated N-acetyltransferase activity.* Mutat Res 1992; 279(2): 83–9.
8. Thompson DC, Eling TE. *Reactive intermediates formed during the peroxidative oxidation of anisidine isomers.* Chem Res Toxicol 1991; 4(4): 474–81.

9. Stiborova M, Miksanova M, Havlicek V, Schmeiser HH, Frei E. *Mechanism of peroxidase-mediated oxidation of carcinogenic o-anisidine and its binding to DNA.* Mutat Res 2002; 500(1–2): 49–66.
10. *Animal Protection Act* of August 21, 1997.Off J Laws 1997, 111, 3445–53 [in Polish].
11. Mahin DT, Lofberg RT. *A simplified method of sample preparation for determination of tritum, carbon-14 or sulfur-35 in blood or tissue by liquid scintillation counting.* Anal Bioch 1966; 16: 500–9.
12. Brij M, Mitrnka H, Rawnsley M. *Clinical biochemical and hematological reference values in normal experimental animals.* New York: Masson Publishing Inc.; 1977.
13. Krzymowski T. *Animals Physiology*. Warsaw: National Agriculture and Forestry Publishers (PWRiL); 1973 [in Polish].