

Genotoxicidad de la furazolidona y la forma libre de su metabolito 3-amino-2-oxazolidona, mediante la prueba de micronúcleos en linfocitos humanos

Genotoxicity of furazolidone and the free form of the metabolite: 3-amino-2-oxazolidone, based on human lymphocyte micronucleus test

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Abstract

The aim of this trial was to assess the genotoxic effects of the main metabolite of furazolidone (3-amino-2-oxazolidone-AOZ), which is usually protein-bound (PB-AOZ). Because PB-AOZ is not available as a tool for biomedical research, the synthetic free form of AOZ (F-AOZ) was used to challenge human lymphocytes in the genotoxic quantification test of induced micronuclei on human lymphocytes. The level of exposure of lymphocytes to F-AOZ was calculated by determining the residual quantity of the Bg-AOZ (from liver and muscle) by HPLC, derived from broilers fed furazolidone included at 0.11% and 0.22% in feed, and allowing a seven day withdrawal time. Then F-AOZ and furazolidone as positive genotoxic group were added at various concentrations higher than the residual level indication to the in vitro preparations diluted both in dimethyl sulfoxide (DMSO) as follows: for furazolidone (FZD) groups of 10 μ M (225 mg/g), 1.0 μ M (225 mg/g), 0.1 μ M (22.5 mg/g), and 0.001 μ M (0.225 mg/g), as well as a negative control group and positive control with DMSO 10⁻³ M (0.130 mg/g) and arsenic 10⁻³ M (0.747 mg/g), respectively; for F-AOZ 0.01 μ M (1.020 mg/g); 0.102 μ M; 0.0005 μ M (0.051 mg/g); and 0.0001 μ M (0.001 mg/g) were tested, having the same controls groups as for FZD. Results show that furazolidone from 10.0 μ M through 0.1 μ M possesses a well defined genotoxic effect. Association frequency, relative risk and ANOVA test showed a statistically significant effect vs the negative control group (P = 0.001; P = 0.03 and P = 0.04, respectively). For F-AOZ the same statistical tests showed that only 0.01 μ M was capable of inducing a genotoxic effect. These results suggest that furazolidone as parent compound is potentially capable of inducing genotoxicity in consumers. In contrast, only the highest concentration of F-AOZ was shown to induce a similar effect. Yet this concentration is well above the expected residual concentration after a 7-day withdrawal period. These results do not support the use of furazolidone in humans as it is now accepted and reveals that F-AOZ is a considerably lower hazard to public health than the parent compound. Yet, lack of evidence of the effect of bound-AOZ in a similar setting precludes further comparisons, but these results suggest that it seems unlikely that PB-AOZ is a real risk to public health. Further studies are warranted.

Key words: FURAZOLIDONE, 3-AMINE-2-OXAZOLIDONE, RESIDUES, GENOTOXICITY, HUMAN LYMPHOCYTES, MICRONUCLEUS TEST.

Resumen

El objetivo de este estudio fue evaluar los efectos genotóxicos del metabolito principal de la furazolidona 3-amino-2-oxazolidona (AOZ) que usualmente se encuentra unido a la proteína (AOZ-UP). Debido a que no se dispone para investigación biomédica de AOZ-UP, se utilizó la forma libre de AOZ (AOZ-L) como desafío genotóxico por medio de la técnica de cuantificación de micronúcleos inducidos en linfocitos humanos. El nivel de exposición de linfocitos a AOZ-libre fue establecido con base en la determinación por cromatografía líquida de alta resolución (CLAR) de los residuos de AOZ-UP encontrados en músculo e hígado de pollos, producidos en forma comercial, expuestos a furazolidona (FZD) por medio del alimento a dosis de 0.11% y 0.22%, permitiendo un tiempo de retiro de 7 días. Se conformaron dos grupos furazolidona (FZD) con las siguientes concentraciones de 10 μ M (225 mg/g), 1 μ M (225 mg/g), 0.1 μ M (22.5 mg/g), y 0.001 μ M (0.225 mg/g), así como el grupo testigo negativo sulfoxido de dimetilo (DMSO) 10⁻³ μ M (0.130 mg/g) y el testigo positivo arsénico 10⁻³ μ M (0.747 mg/g).

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Para AOZ-libre las concentraciones fueron 0.01 μM (1.020 mg/g); 0.001 μM (0.102 mg/g); 0.0005 μM (0.051 mg/g); y 0.0001 μM (0.001 mg/g) con los mismos grupos testigo. Los resultados muestran que la furazolidona a concentraciones de 1.0 μM y 0.1 μM posee un efecto genotóxico bien definido. El grado de asociación se calculó por medio del riesgo relativo y prueba de ANDEVA, que mostró el efecto estadísticamente significativo al compararlo con el grupo testigo negativo ($P = 0.001$; $P = 0.03$ y $P = 0.04$, respectivamente). Para AOZ-L las mismas pruebas estadísticas mostraron que sólo la concentración 0.01 μM era capaz de inducir un efecto genotóxico. Estos resultados sugieren que la furazolidona como sal pura es potencialmente capaz de inducir efectos genotóxicos en humanos, en los que no se apoya su uso. En contraste, sólo la concentración más alta de AOZ-L mostró un efecto similar, pero dicha concentración es mayor que la encontrada como residual a los siete días de retiro y puede considerarse como un peligro mucho menor para la salud pública que el compuesto progenitor. Dada la falta de evidencia científica del efecto genotóxico del AOZ-UP no se pueden realizar comparaciones adicionales con lo obtenido aquí para AOZ-L, pero parecería poco probable calificar a los residuos de AOZ-UP como peligros reales para la salud pública, por lo que se requieren pruebas adicionales.

Palabras clave: FURAZOLIDONA, 3-AMINO-2-OXAZOLIDONA, RESIDUOS, GENOTOXICIDAD, LIMFOCITOS HUMANOS, PRUEBA DE MICRONUCLEOS.

Introduction

Worldwide use of nitrofurans in productive animals has been objected.¹⁻⁵ For instance, the FDA (Food and Drug Administration) of the United States of America considers that its commercialization must be banned, since it can generate carcinogenic residues in animal tissues.¹⁻⁶ The Joint FAO/WHO Expert Committee on Food Additives (JEFCA) considers them genotoxic and capable of increasing the incidence of malignant tumors in mice and rats.⁷ Therefore, the use of the mentioned antibacterial family for food production has been banned by the majority of import food markets. Nevertheless, the JEFCA points out that information on carcinogenic potential effect of nitrofurans metabolites, for instance, furazolidone and in particular the metabolite 3-amino-2-oxazolidone (AOZ), is incomplete, for which it is necessary to determine if protein-bound residues (AOZ-PB) are indeed genotoxic.⁸⁻¹⁴ Unfortunately, there are no AOZ-PB forms available for research. It is postulated that it may interfere with food safety standards, since it may persist in the destined animal for human consumption or its by-products for long periods after treatment is ended, and because there is the potential that, with digestion, bound residues may be released, giving rise to potentially toxic forms.^{15,16} In contrast, there is evidence indicating a low AOZ-PB genotoxicity.¹⁵

Although there is evidence that there are two different criteria in regard to nitrofurans dangerousness, the *Codex Alimentarius* has not established an admissible daily intake value (ADI), which has frequently generated extreme criteria to be proposed. For instance, for the European Union, the required detection limit for accepting analytical results is a maximum of 1 ng/g (1 ppb) and it is called: required minimum functionality. If an analytic method does not reach this sensitivity, it is considered inadequate. In regard to Ridascreen®,* it is an immunoassay based on a commercial quantita-

Introducción

Se ha objetado el uso de nitrofuranos en animales productivos en todo el mundo.¹⁻⁵ Por ejemplo, la (FDA) Food and Drug Administration de los Estados Unidos de América considera que se debe prohibir su comercialización dado que pueden generar residuos carcinogénicos en tejidos animales.¹⁻⁶ El denominado *Joint FAO/WHO Expert Committee on Food Additives* (JEFCA) los considera genotóxicos y capaces de aumentar la incidencia de tumores malignos en ratones y ratas.⁷ De tal suerte que el uso de dicha familia de antibacterianos para la producción de alimentos ha sido prohibido por la mayoría de los mercados importadores de alimentos. No obstante, el JEFCA puntualiza que la información sobre el efecto potencial carcinogénico de los metabolitos de los nitrofuranos, por ejemplo de la furazolidona y en particular del metabolito 3-amino-2-oxazolidona (AOZ), es incompleta, por lo que se requiere determinar si los residuos ligados a proteínas (AOZ-UP) son en realidad genotóxicos.⁸⁻¹⁴ Desafortunadamente no hay formas de AOZ-UP disponibles para investigación. Se postula que pueden interferir con la seguridad alimentaria dado que pueden persistir en el animal destinado al consumo humano o sus productos por largos periodos después de finalizar el tratamiento, y porque existe el potencial de que, con la digestión, sean liberados de la proteína con la que vienen unidos, dando lugar a formas potencialmente tóxicas.^{15,16} En contraste, hay evidencia que indica una baja toxicidad y genotóxica del AOZ-UP.¹⁵

Aunque es evidente que existen dos corrientes de pensamiento con respecto a la peligrosidad de los nitrofuranos, el *Codex Alimentarius* no ha establecido un valor de ingestión diaria admisible (IDA), lo que ha generado que se propongan criterios a menudo extremos. Por ejemplo, para la Unión Europea, el límite de detección exigido para aceptar resultados analíticos es como máximo 1 ng/g (1 ppb) y se le denomina: lími-

tive test that can detect 10 ppt (0.01 ppb). In contrast with the last cipher, in genotoxicity tests carried out in experimental animals, much higher doses or concentrations of these potential carcinogens and during relatively short periods were used. This has generated speculations on predictive value of risk for humans exposed to much lower doses and for often intermittent exposure periods and of variable duration. In this test, lymphocytes used as genotoxicity markers are utilized and is extrapolated, apparently with great certainty, to human populations exposed to xenobiotics.¹⁷ This test is based on chromosome breakage, heritable mutations or induction of genetic mutations.¹⁸ That is, the micronuclei are isolated chromosome fragments or complete chromosomes due to an aberration in mitotic or meiotic division that spontaneously keep them out of the nucleus. Its presence increases significantly when the organism study is exposed to clastogenic and aneuploidogenic agents, which enter to daughter cells after cytokinesis.^{19,20}

Given the apparent discrepancy on information regarded to genotoxicity of furazolidone metabolite residues [mainly 3-amino-2-oxazolidone (AOZ), free or protein-bound], it was considered necessary to carry out an assay to evaluate the potential that this free form of metabolite can have in the matter, and its comparison with the parent compound (furazolidone), using one of the most sensitive tests: micronuclei in human lymphocytes, but at initial concentrations found in broiler meat and viscera exposed to non-toxic high doses of furazolidone for long periods and increasing these concentrations in \log_{10} multiples.

Material and methods

Three hundred Ross 308 chickens, divided in three groups of 100 chicks each were used. Birds were subjected to a conventional production cycle; one group initially received 110 g of furazolidone/ton of feed for 28 days, and a second group received 220 g of furazolidone/ton of feed for 10 days; doses of 55 g/ton were kept for both groups until week six of age. A non-medicated control group was formed, handled the same as the rest. Feed was removed from both groups seven days before chickens were slaughtered.²¹

Once the productive cycle of the 3 groups of birds was concluded, they were slaughtered by means of physical methods according to NOM-009-Z00-1994²² and samples of muscular tissue from leg and liver were obtained. They were identified and frozen at -20°C until their analysis. From each group, 50 birds were randomly sampled, including control group.

The analytical technique was based on high-performance liquid chromatography (HPLC) as developed

te mínimo de funcionamiento exigido. Si un método analítico no alcanza esta sensibilidad, no se le considera apto. En cuanto a Ridascreen®,* es una prueba cuantitativa comercial basada en inmunoensayo que puede detectar 10 ppt (0.01 ppb). En contraste con la cifra anterior, en las pruebas de genotoxicidad en animales de laboratorio se utilizan dosis o concentraciones mucho más altas de estos potenciales carcinógenos y durante periodos de exposición relativamente cortos. Esto ha generado especulaciones sobre el valor predictivo del riesgo para los humanos expuestos a dosis mucho más bajas y durante periodos de exposición a menudo intermitente y de duración muy variable. En ese sentido, cabe destacar a la prueba de micronúcleos en linfocitos humanos. En esta prueba se utilizan linfocitos como marcadores de genotoxicidad y se extrapola, aparentemente con mayor certeza, a poblaciones humanas expuestas a xenobióticos.¹⁷ Esta prueba se basa en el rompimiento cromosómico, mutaciones heredables o inducción de mutaciones genéticas.¹⁸ Esto es, los micronúcleos son fragmentos de cromosomas o cromosomas completos aislados debido a una división mitótica o meiótica errónea que de manera espontánea quedan fuera del núcleo. Su presencia se incrementa significativamente cuando el organismo a estudiar se expone a agentes clastógenos y aneuploidogénicos, que ingresan a las células hijas después de la citocinesis.^{19,20}

Dada la aparente discrepancia en la información con lo que respecta a la genotoxicidad de los residuos de metabolitos de furazolidona (principalmente 3-amino-2-oxazolidona [AOZ], libre o unida a proteínas), se consideró necesario realizar un ensayo para evaluar el potencial que este metabolito en forma libre puede tener al respecto, y su comparación con el fármaco progenitor (furazolidona), utilizando una de las pruebas más sensibles: micronúcleos en linfocitos humanos, pero a concentraciones iniciales encontradas en vísceras y carne de pollo de engorda expuestos a dosis altas no tóxicas de furazolidona por largos periodos y elevando dichas concentraciones en múltiplos de \log_{10} .

Material y métodos

Se emplearon 300 pollos de la estirpe Ross 308, divididos en tres grupos de 100 pollos cada uno. Las aves se sometieron a un ciclo de producción de forma convencional; a un grupo se le medicó inicialmente 28 días con dosis de 110 g de furazolidona/ton de alimento, y un segundo grupo se medicó a dosis de 220 g de furazolidona/ton de alimento por 10 días; se mantuvieron las dosis de 55g/ton en ambos grupos hasta

*®Ridascreen. Nitrofurans (AOZ) R3703 r-biopharm, Germany.

by Angellini *et al.*²³ and validated for determination of nitrofurans residues in muscle tissues stabilized at pH 6.0. Residues were extracted with acetonitrile, the sample was purified by liquid-liquid partition between ethyl acetate and acetonitrile and extract was concentrated. Drug residues were dissolved in LC mobile phase [0.01M sodium acetate buffer (pH 4.5)- acetonitrile (70:30)], filtered and determined by HPLC, for which a reverse-phase C18 column (Hypersil ODS) at 35°C was used, utilizing a UV-visible detector* with diode arrangement and at 365 nm. Retention times and UV spectra of the peaks of the sample were compared with furazolidone standard patterns** and AOZ.*** The AOZ metabolite quantity detected (protein-bound) in the 110 g/ton dose groups was 0.108 mg/g \pm 0.3 mg/g, and the group receiving 220 g/ton was 0.135 mg/g \pm 0.178 mg/g. This last group showed greater AOZ quantity either in muscle or liver ($P < 0.05$). These data were useful as guideline values to carry out the micronuclei bioassays (MN), utilizing the found average of 0.464 mg/g as middle point, increasing and decreasing concentrations for bioassays with lymphocytes. For furazolidone, greater or smaller values at a medium concentration (2.25 mg/g) consistent with the ones that would be achieved in tissues, given a therapeutic dose in humans, were used.

The MN bioassays were carried out at the Laboratorio de Citogenética of the Instituto Nacional de Cancerología, according to the described by Fenech.^{24,25} The lymphocytes were obtained from whole blood from 10 clinically healthy, non-smoker and non-alcohol consumer volunteers, whose ages ranged between 20 to 45 years. It was proven by anamnesis that they did not receive treatment during 30 days previous to donation. The following experimental groups were formed: a) furazolidone Group: with human lymphocytes exposed to the following furazolidone concentrations: 10 μ M (22 mg/g), 1 μ M (225 mg/g), 0.1 μ M (2.25 mg/g), 0.001 μ M (0.225 mg/g). Dimethyl sulfoxide (DMSO) 10⁻³ μ M (1.303 mg/g) was used as negative control group, and arsenic 10⁻³ μ M (0.749 mg/g) as positive control group; b) free AOZ Group: with human lymphocytes exposed to free AOZ* concentrations: 0.01 μ M (1.029 mg/g); 0.001 μ M (0.102 mg/g); 0.005 μ M (0.0510 mg/g); 0.0001 μ M (0.001 mg/g). Also, dimethyl sulfoxide (DMSO) 10⁻³ μ M (1.303 mg/g) was used as negative control group, and arsenic 10⁻³ μ M (0.749 mg/g) as positive control group.

The evaluation of MN was carried out in 1 000 binuclei cells (BNC), lecture was done by duplicate and masking the treatments. Criteria used to identify micronuclei in BNC, are accepted in international studies²⁰ and are summarized as follows:

For identifying BNC the cell must have two round or oval nuclei, nuclei must not be connected by chro-

la sexta semana de edad. Se formó un grupo testigo no medicado, manejado de la misma manera que los demás. Siete días antes del sacrificio se retiró el alimento medicado en ambos grupos.²¹

Una vez concluido el ciclo productivo de las aves de los 3 grupos se les sacrificó por métodos físicos de acuerdo con la NOM-009-Z00-1994²² y se obtuvieron muestras de masa muscular de pierna e hígado. Se les identificó y congeló a -20°C hasta su análisis. De cada grupo se muestrearon aleatoriamente 50 aves, incluyendo el grupo testigo.

La técnica analítica se basó en cromatografía líquida de alta resolución (CLAR) de la forma desarrollada por Angellini *et al.*²³ y validada para la determinación de residuos de nitrofuranos en muestras de tejidos musculares estabilizadas a pH 6.0. Los residuos se extrajeron con acetonitrilo, se purificó la muestra por partición líquido-líquido entre el acetato de etilo y acetonitrilo, y se concentró el extracto. Los residuos del fármaco fueron disueltos en la fase móvil [(0.01 M tampón de acetato de sodio (pH 4.5)-acetonitrilo (70:30)], se filtró y se determinó por CLAR, para lo cual se empleó una columna C18 de fase inversa (Hypersil ODS) a 35°C, usando un detector de red UV-visible* con arreglo de diodos y a 365 nm. Los tiempos de retención y el espectro ultravioleta de picos de las muestras se compararon con los de los patrones estándar de furazolidona** y AOZ.*** La cantidad detectada del metabolito AOZ (unido a proteína) en el grupo dosificado a 110 g/ton fue de 0.108 mg/g \pm 0.3 mg/g, y en el grupo que recibió 220 g/ton fue de 0.135 mg/g \pm 0.178 mg/g. Este último grupo mostró una mayor cantidad de AOZ tanto en músculo como en hígado ($P < 0.05$). Estos datos sirvieron como valores directrices para realizar los bioensayos de micronúcleos (MN), utilizando el promedio encontrado de 0.464 mg/g como punto medio, aumentando y disminuyendo las concentraciones para los bioensayos con linfocitos. Para la furazolidona se usaron valores mayores y menores a una concentración media (2.25 mg/g) congruente con las que se lograrían en tejidos, dada una dosis terapéutica en humanos.

Los bioensayos de MN se realizaron en el laboratorio de Citogenética del Instituto Nacional de Cancerología, conforme a lo descrito por Fenech.^{24,25} Los linfocitos se obtuvieron a partir de sangre entera de 10 donadores voluntarios, clínicamente sanos, no fumadores ni consumidores de alcohol, con un rango de edad entre los 20 y 45 años. Se constató por anamnesis que no recibieron tratamiento durante los 30 días previos a la donación. Se formaron los siguientes grupos experimentales: a) Grupo furazolidona: con células de

*Sonntek, Inc. 125 Pleasant Avenue. Upper Saddle River NJ. USA 07458.

**SIGMA-ALDRICH, No de cat 46297-250MG Furazolidona.

***No. cat. 33347-50MG-R AOZ, VETANAL analytical standard.

matin bridges, nuclei may be at early prophase, nuclei must have a similar size (not necessarily identical), nuclei may be partially superposed and have a well preserved cytoplasm, micronuclei must have a round or oval form, 1/2 to 1/5 size of the nucleus, not refringent, they must present equal color and intensity as the main nucleus, they must not touch any nucleus, they must present same condensation as the nucleus.

The results were processed by means of STATA 11.01 statistical package.** The descriptive statistics were used to calculate measures of central tendency and dispersion and inferential statistics were used to measure degree of association of MN by relative risk and etiologic fraction, and an analysis of variance was performed to find the dose that will cause the effect study, according to the suggested by Hernandez and Lopez.²⁶

Results

Figure 1 shows, as frequency bars, the mean \pm 1 standard deviation (SD) of micronuclei number observed for treated lymphocytes either with furazolidone or free AOZ, having control group as reference. In that same figure, letters indicate the values that presented significant differences.

In regard to groups treated with furazolidone, concentrations that had greater association degree with micronuclei production were: 1 μ M, 10 μ M and 0.001 μ M of furazolidone (22.5 μ g/g, 225 μ g/g and 0.225 μ M). The concentration of 10 and 0.1 μ M showed relative risk of 1.36; that is, lymphocytes exposed to this concentration had 36% more risk to develop micronuclei in regard to the unexposed. An etiologic fraction was obtained in both concentrations; that is, 26.7% of MN development in exposed population is due to this concentration. The concentration of 1 μ M showed a relative risk of 1.45, meaning that those lymphocytes exposed to 1 μ M had 45% more risk of micronuclei development in regard to the unexposed, with an etiologic fraction of 0.313. In 0.1 μ M concentration, the relative risk was 1.09 with an etiologic fraction of 0.004; based on these data this association was at random. By means of Levene's test, variance homogeneity was detected ($P < 0.001$), for which an ANOVA test ($P < 0.001$) was performed. It is concluded that there are statistically significant differences in treatments; based on it, matched-pairs t-test on the data was performed, to determine which treatment or treatments make the difference. It was observed that there is only effect of treatment on the following concentrations: DMSO *vs* 1.0 μ M ($P = 0.03$) and DMSO *vs* 0.1 μ M ($P = 0.04$).

In regard to AOZ metabolite, it was observed that the concentration that had the greatest degree of as-

linfocitos humanos expuestas a las siguientes concentraciones de furazolidona: 10 μ M (225 mg/g), 1 μ M (225 mg/g), 0.1 μ M (2.25 mg/g), 0.001 μ M (0.225 mg/g). Se utilizó sulfóxido de dimetilo (DMSO) 10^{-3} μ M (1.303 mg/g) como testigo negativo de grupo, y arsénico 10^{-3} μ M (0.749 mg/g) como testigo positivo de grupo. b) Grupo AOZ-L: con células de linfocitos humanos expuestas a concentraciones de AOZ-no unido a proteína-libre:* 0.01 μ M (1.029 mg/g); 0.001 μ M (0.102 mg/g); 0.0005 μ M (0.0510 mg/g); 0.0001 μ M (0.001 mg/g). También se uso sulfóxido de dimetilo (DMSO) 10^{-3} μ M (1.303 mg/g) como testigo negativo de grupo, y arsénico 10^{-3} μ M (0.749 mg/g) como testigo positivo de grupo.

La evaluación de los MN se realizó en 1000 células bi-nucleadas (CBN), la lectura se efectuó por duplicado y enmascarando los tratamientos. Los criterios que se emplearon para identificar micronúcleos en CBN, son los aceptados en estudios internacionales²⁰ y que se resumen a continuación:

Para identificar CBN la célula debe tener dos núcleos redondos u ovals, los núcleos no deben estar conectados por puentes de cromatina, los núcleos pueden estar en profase temprana, los núcleos deben tener un tamaño similar (no necesariamente idéntico), los núcleos pueden estar parcialmente sobrepuestos y tener citoplasma bien conservado, los micronúcleos deberán tener forma redonda u oval, tamaño 1/2 a 1/5 del tamaño de los núcleos, no deben ser refringentes, deben presentar igual color e intensidad que el núcleo principal, no deben tocar ninguno de los núcleos, deben presentar la misma condensación que los núcleos.

Los resultados se procesaron por medio del paquete estadístico STATA 11.01.** Se empleó la estadística descriptiva para calcular las medidas de tendencia central y de dispersión y se utilizó la estadística inferencial para medir el grado de asociación para inducir MN por medio del riesgo relativo y fracción etiológica, y se realizó un análisis de varianza para encontrar la dosis que produzca el efecto estudiado de acuerdo con lo sugerido por Hernández y López.²⁶

Resultados

En la Figura 1 se presentan como barras de frecuencia la media \pm 1 desviación estándar (DE) del número de micronúcleos observados para los linfocitos tratados tanto con furazolidona como con AOZ-L, teniendo como referencia los grupos testigo. En esa misma

*SIGMA-ALDRICH No. Catalogo 33347.

**Stata Corp. 4905 Lakeway Drive, College Station, Texas 77845. USA.

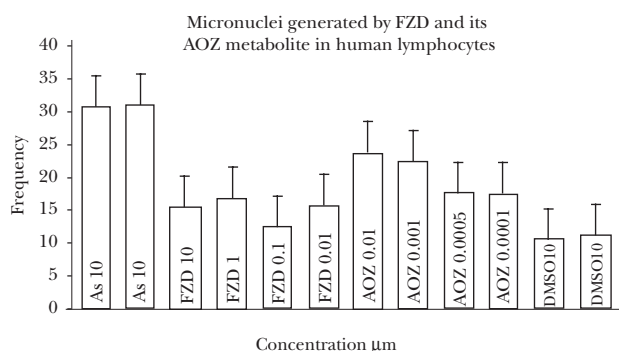


FIGURA 1. Frecuencia ($X \pm 1$ DE) del número de micronúcleos presentes en linfocitos humanos de donadores sanos, estimulados con furazolidona o con 3-amino-2-oxazolidona (AOZ). Se presentan los valores de los testigos positivo con arsénico (As) y negativo con dimetil-sulfóxido (DMSO).

FIGURE 1. Frequency ($X \pm 1$ SD) of micronuclei number present in human lymphocytes from healthy donors, stimulated with furazolidone or with 3-amino-2-oxazolidone (AOZ). Arsenic (As) positive and dimethyl-sulfoxide (DMSO) negative controls are depicted.

sociation with micronuclei production was 0.01 μ M (1.029 mg/g), with a relative risk of 2.15 and an etiologic fraction of 0.61. For concentrations of 0.001 μ M an RR of 2.33 was obtained with an etiologic fraction of 0.571, for concentrations of 0.005 μ M an RR of 1.44 was obtained with an etiologic fraction of 0.308 and for the concentration of 0.0001 an RR of 1.77 was obtained with an etiologic fraction of 0.437. Variance homogeneity ($P = 0.036$) followed by ANOVA ($P = 0.044$) was detected by Levene's test. The subsequent Student's *t*-tests revealed that when DMSO with 0.01 μ M of AOZ were contrasted, there was statistically significant difference ($P = 0.03$).

Discussion

Cytokinesis-block micronucleus technique by means of cytochalasin B (Cyt-B) identifies cells that have been once divided in culture and they allow assessing the generated micronuclei frequency only by conditions study.²⁵ By means of this methodology, cells are observed as binucleic due to the inhibitory effect of Cyt-B on cytokinesis, and without altering karyokinesis. This technique is described as a sensitive tool for genetic damage detection, aside from being rapid, it is easily to apply to different cellular types and it can also be substituted by chromosome aberration analysis or sister chromatid exchange.²⁰ By means of the MN technique it is possible to discriminate micronuclei origin between clastogenic and aneugenic using antikinetochore antibodies or fluorescent probes for centromere and telomere regions, giving the possibility to discern the action mechanism of diverse genotoxic agents.⁷

figura se destacan mediante literales los valores que presentaron diferencias significativas.

Para los grupos tratados con furazolidona, las concentraciones que tuvieron el mayor grado de asociación con la producción de micronúcleos fueron 1 μ M, 10 μ M y 0.001 μ M de furazolidona (22.5 μ g/g, 225 μ g/g y 0.225 μ M). La concentración de 10 y 0.1 μ M presentaron un riesgo relativo de 1.36, es decir los linfocitos expuestos a esta concentración tuvieron 36% más riesgo de desarrollar micronúcleos con respecto a los no expuestos. En ambas concentraciones se obtuvo una fracción etiológica de 0.267; es decir, el 26.7% del desarrollo de MN en la población expuesta se debe a esta concentración. La concentración 1 μ M presentó un riesgo relativo de 1.45, es decir, aquellos linfocitos que se expusieron a la dosis de 1 μ M tuvieron 45% más riesgo de desarrollar micronúcleos con respecto a los no expuestos, con una fracción etiológica de 0.313. En la concentración 0.1 μ M el riesgo relativo fue de 1.09 con una fracción etiológica de 0.004 con base en estos datos dicha asociación se debió al azar. Mediante la prueba de Levene se detectó homogeneidad de varianzas ($P = 0.01512$), por lo que se hizo una prueba de ANDEVA ($P < 0.001$). Se concluye que hay diferencias estadísticamente significativas en los tratamientos, con base en ello se hizo la prueba de "t" de Student entre pares de datos, para saber qué tratamiento o tratamientos determinan las diferencias. Se detectó que sólo hay efecto de tratamiento en las concentraciones DMSO vs. 1.0 μ M ($P = 0.03$) y DMSO vs. 0.1 μ M ($P = 0.04$).

Con respecto al metabolito AOZ, se detectó que la concentración que mayor grado de asociación tuvo con la producción de micronúcleos fue la de 0.01 μ M (1.029 mg/g), con un riesgo relativo de 2.51 y una fracción etiológica de 0.61. Para las concentraciones de 0.001 μ M se obtuvo un RR de 2.33 con un fracción etiológica de 0.571, para concentración de 0.005 μ M se obtuvo un RR de 1.44 con una fracción etiológica de 0.308 y para la concentración de 0.0001 se obtuvo un RR de 1.77 con una fracción etiológica de 0.437. Mediante la prueba de Levene se detectó homogeneidad de varianzas ($P = 0.036$) seguida de ANDEVA ($P = 0.044$). Las subsecuentes "t" de Student revelaron que sólo cuando se contrastaron DMSO con 0.01 μ M de AOZ hubo diferencia estadísticamente significativa ($P = 0.03$).

Discusión

La técnica de micronúcleos con el bloqueo de la citocinesis por medio de la citocalasina B (Cyt-B) identifica a las células que se han dividido una vez en cultivo y de esta manera permiten estimar la frecuencia de micronúcleos generados únicamente bajo las condi-

Considering the sensitivity and specificity of MN test, it is feasible to postulate that the obtained data confirm the FZD capacity for inducing a well defined genotoxic effect on concentrations of 0.1 and 10 μ M for seven days (22.5 μ g/g and 2 250 μ g/g), and in this study the results of this assay coincide with the majority of the authors²⁷⁻⁴⁶ although they use different methodologies. In counterpart, Enniga and Weterings⁴⁷ report negative genotoxicity results evaluated by micronuclei test but in lymphocytes of Swiss CD-1 mice or Swiss Webster mice, respectively and administering FZD per oral via at doses of 100 to 500 mg/kg.⁴⁹

In contrast, only the highest free AOZ form concentration (1.029 mg/g) was the one that showed MN significant induction. In this sense Hoogenboom *et al.*¹¹ coincide, who described AOZ mutagenic effects and formation of protein adducts, using microsome mutagenicity tests with *Salmonella* TA 1535 and TA 100 strains with metabolic activation of rat liver S9 fraction.

They also obtained a positive chromosome aberration test response in humans and with erythrocytes in bone marrow micronuclei test of mice treated with protein-bound AOZ of high molecular weight, but administering exaggerated quantities by direct intraperitoneal injection. Authors conclude that there is release and systemic absorption of AOZ and that it could be potentially mutagenic. The results here obtained and those documented by Hoogenboom *et al.*¹¹ contrast with the described by Scheres,⁵⁰ who reports that negative genotoxicity results using free form of AOZ but with Ames test with and without rat liver S9 fraction, in *Salmonella* serovar Typhimurium and in concentrations of 100-500 μ g/ml of AOZ.

The obtained in this assay and the reported by Scheres⁵⁰ cannot be compared with studies using protein-bound AOZ, as the one of Auro *et al.*⁵¹ who evaluated the genotoxicity of food with AOZ-PB residues in gynogenetic fish. They postulate that it is unlikely to consider protein-bound AOZ as a danger in the trophic chain. In that sense, it is then evident that it would be necessary to carry out a structural chemical determination of the way in which AOZ binds to different proteins and the dynamic of its release, if it is the case, prior to digestive process²⁷ in order to determine its potential dangerousness.

It must be pointed out that genotoxicity was only observed in this study using three times more AOZ concentration than the reported in literature as possible contaminant of meat by-products after a seven-day withdrawal period.^{50,52-54} This situation has artificial components since, in natural conditions, AOZ is not found in its free form, but also without withdrawal time it is unlikely to find those quantities in tissues. Therefore, although results here exposed suggest genotoxicity by free AOZ, the rate of exposure to this metabolite

ciones del estudio.²⁵ Con esta metodología las células se observan como binucleadas debido al efecto inhibitorio de la Cyt-B sobre la citocinesis, y sin alterar la cariocinesis. A esta técnica se le describe como una herramienta sensible para la detección de daño genético, además de ser rápida, de fácil aplicación a diferentes tipos celulares e incluso puede sustituir el análisis de aberraciones cromosómicas o intercambio de cromátidas hermanas.²⁰ Con la técnica de MN también es posible discriminar el origen de los micronúcleos entre clastogénicos o aneugénicos empleando anticuerpos anticinetocoro o bien sondas fluorescentes para centrómeros y telómeros, lo que da la posibilidad de discernir el mecanismo de acción de diversos agentes genotóxicos.⁷

Considerando la sensibilidad y especificidad de la prueba de MN es factible postular que los datos obtenidos confirman la capacidad de la FZD para inducir un efecto genotóxico bien definido en concentraciones de 0.1 y de 10 μ M por 7 días (22.5 μ g/g y 2250 μ g/g) y en este sentido, los resultados de este ensayo coinciden con la mayoría de los autores²⁷⁻⁴⁶ aunque utilizando diferentes metodologías. En contraparte, Enniga y Weterings⁴⁷ y Paik,⁴⁸ informan de resultados negativos a genotoxicidad evaluada mediante la prueba de micronúcleos, pero en linfocitos de ratones Swiss CD-1 o en ratones Swiss Webster, respectivamente y administrando FZD por vía oral a dosis de 100 a 500 mg/kg.⁴⁹

En contraste, sólo la concentración más alta de AOZ libre (1.029 mg/g) fue la que mostró la inducción significativa de MN. En este sentido coinciden Hoogenboom *et al.*,¹¹ quienes describen efectos mutagénicos del AOZ y formación de aductos en proteínas, empleando pruebas de mutagenicidad microsómica con cepas de *Salmonella* TA 1535 y TA 100 con activación metabólica de la fracción S9 de hígado de rata.

También obtuvieron una respuesta positiva en la prueba de aberraciones cromosómicas en linfocitos humanos y con eritrocitos en la prueba de micronúcleos en médula ósea de ratones tratados con AOZ ligado a proteína de alto peso, pero administrando cantidades exageradas mediante inyección intraperitoneal directa. Los autores concluyen que hay liberación y absorción sistémica de AOZ y que pudiera ser potencialmente mutagénica. Los resultados aquí obtenidos y los documentados por Hoogenboom *et al.*¹¹ contrastan con lo detallado por Scheres,⁵⁰ quien informa de resultados negativos a genotoxicidad utilizando AOZ libre pero con la prueba de Ames con y sin fracción S9 de hígado de rata, en *Salmonella* Typhimurium y en concentraciones de 100-500 μ g/ml de AOZ.

Tanto lo obtenido en este ensayo como lo informado por Scheres⁵⁰ no pueden compararse con estudios que utilizan AOZ ligada a proteínas, como el de Auro *et al.*⁵¹ que evaluaron la genotoxicidad de alimento con

allows to question if AOZ-PB real fraction is indeed a hazard to public health and questions, with the objective to safeguard public health, the rejection validity of exported meat, generated by analytical tests that detect few picograms of AOZ in animal tissues (ppt), for instance Ridascreen®.* Moreover, given the proven genotoxicity of FZD, it results contrasting that while veterinary medicine condemns its use, it is allowed that this drug be prescribed for human medicine, giving rise to question health measures as, for example, to condemn for disposal meat products containing ppt of protein-bound AOZ and whose genotoxicity is still debatable.

Referencias

1. FOOD AND DRUG ADMINISTRATION. Department of Health, Education, and Welfare Federal Register Vol. 41, No. 94-Thursdays, May 13, 1976.
2. KESSLER DA. Commissioner of Food and Drugs. Federal Register Doc 91-20219.
3. FEDERAL REGISTER (FR) Vol. 55 No. 164. August 23, 1991. Pages 41797-41913
4. FEDERAL REGISTER (FR) Vol. 62, No. 99. Thursday, May, 1997. Rules and Regulations. Page 27944.
5. FEDERAL REGISTER: February 6, 2002 (Volume 67, Number 25. Rules and Regulation. Page 5470-5471. From the Federal Register [online] via GPO Acces: URL: <http://www.wais.access.gpo.gov>. DOCID: fr06fe02-8.
6. EAGLE BARNEY. Food safety regulations of the European Union on food contaminants derived from veterinary medical products. *Maygar Allatorvosok Lapja* 1995; 50: 275-280.
7. VAN KOTEN-VERMEULEN JEM, WOUTERS MFA, VAN LEEUWEN FXR. Furazolidone. In 774. *Furazolidone*. Who Food Additives Series 31. Ipc. Inchem. Serial on line (cited 2003 Nov 6). Available from: <http://www.inchem.org/documents/jecfa/jecmono/v31je06.htm>.
8. MCCRAKEN RJ, KENNEDY DG. Determination of the furazolidone metabolite 3-amino-2-oxazolidone, in porcine tissues using liquid chromatography thermospray mass spectrometry and the occurrence of residues in pigs produced in Northern Ireland. *J Chromatogr B Biomed Sci Appl* 1977; 691: 87-94.
9. COOPER KM, ELLIOTT CT, KENNEDY DG. Detection of 3-amino-2-oxazolidone (AOZ), a tissue bound metabolite of the nitrofurans furazolidone in prawn tissue by enzyme immunoassay. *Food Addit Contam* 2004; 841-848.
10. HOOGENBOOM LA, VAN KAMMEN M, BERGHMANS M, KOEMAN, KUIPER H. The use of pig hepatocytes to study the nature of protein bound metabolites of furazolidone; a new analytical method for their detection. *Food Chem Toxicol* 1991; 29: 321-328.
11. HOOGENBOOM LA, VAN BRUCHEM G, SONNE K, ENNIGA IC, VAN RHIJN JA, HESKAMP H *et al*. Absorption of a mutagenic metabolite released from protein-bound residues of furazolidone. *Environ Toxicol Pharmacol* 2002; 11: 273-287.
12. MCCRAKEN RJ, MCCOY MA, KENNEDY DG. Evaluation of the residues of furazolidone and its metabolite, 3-amino-2-oxazolidone (AOZ) in eggs. *Food Addit Contam* 2001, 18; 11: 954-959.
13. CONNEELY A, NUGENT A, O'KEEFFE M. Use of solid phase extraction for the isolation and clean-up of a derivatised furazolidone metabolite from animal tissues. *Analyst* 2002; 127: 705-709
14. CONNEELY A, NUGENT A, O'KEEFFE M, MULDER PP, VAN RHIJN JA, KOVACSICSL *et al*. Isolation of bound residues of nitrofurans drugs from tissue by solid-phase extraction with determination by liquid chromatography with UV and tandem mass spectrometric detection. *Anal Chem Acta* 2003, 483: 91-98.
15. LEITNER A, ZOLLNER P, LINDNER W. Determination of the metabolites of the nitrofurans Antibiotics

*http://www.sceti.co.jp/medical/PdfFiles/rbo/R3701_Nitrofurame_AOZ.pdf

- in animal tissue by high-performance liquid chromatography-tandem mass spectrometry. *J Chromatogr A*, 2001; 939: 49-58.
16. FOOD BRAND-FOOD AND DRUG ADMINISTRATION. Bound residues and nitrofurans detection. Development of rapid multi-residue screening test and definitive multi-residue methods of the nitrofurans to monitor the effectiveness of the EU ban on the use of. Food, Nutrition and Health. [Serial online: 2000 August] [Cited 2005 Oct 26] Available from: <http://www.afsni.ac.uk/foofbrand/>
 17. ALBERTINI RJ, ANDERSON D, DOUGLAS GR, HAGMAR L, HEMMINKI K, MERLO F. IPCS Guidelines for the Monitoring of Genotoxic Effects of Carcinogens in Humans. *Mutat Res*, 2000; 463: 111-172.
 18. RAMÍREZ T, BENITEZ BL, OSTROSKY P, HERRERA LA. *In vitro* effects of albendazole and its metabolites on the cell proliferation kinetics and micronuclei frequency of simulated human lymphocytes. *Arch Med Res* 2001; 32:119-122.
 19. KLEE S, BAUMUNG I, KLUGE R, UNGEMACHOE K, HORNE FE, O'KEEFFEOE M *et al*. A contribution to safety assessment of veterinary drug residues: *in vitro/ex vivo* studies on the intestinal toxicity and transport of covalently bound residues. *Xenobiotica* 1999;29: 641-654.
 20. RAMIREZ HT. Efectos de la S-Adenosil-L-Metionina sobre la genotoxicidad *in vitro* inducida por diversos xenobióticos (tesis de maestría). México DF: Facultad de Ciencias. Universidad Nacional Autónoma de México, 2003.
 21. BARRAGÁN H EA. Evaluación del efecto genotóxico del metabolito 3-Amino-2-Oxazolidona. Un contaminante químico, en carne y vísceras de pollo destinados al consumo humano (tesis doctoral). Cuernavaca (Morelos) México: Instituto Nacional de Salud Pública, 2006.
 22. DIARIO OFICIAL DE LA FEDERACIÓN. Norma Oficial Mexicana NOM-009-Z00-1994. Proceso sanitario de la carne. Secretaria de Agricultura y Recursos Hidráulicos. Diario Oficial de la Federación. 11/16/1994.
 23. ANGELLINI NM, RAMPINI O.D, MUGICA H. Liquid chromatographic determination of nitrofurans residues in bovine muscle tissues. *J AOAC Inter* 1997; 80: 481-485.
 24. FENECH M, MARLEY AA. Cytokinesis-Block Micronucleus Method in Human lymphocyte: effect of *in vivo* ageing and low dose X-irradiation. *Mutat Res* 1986; 161: 193-198.
 25. FENECH M. The *in vitro* micronucleus technique. *Mutat Res* 2000; 455: 81-85.
 26. HERNÁNDEZ AM, LÓPEZ MS. Capítulo II Diseño de Estudios epidemiológicos. En: HERNÁNDEZ AM, editor. Epidemiología diseño y análisis de estudios. México DF: Ed Instituto Nacional de Salud Pública, y Médica Panamericana, 2007: 17-32.
 27. JAGANNATH DR, BRUSICK DJ. Mutagenicity evaluation of Furazolidone WIL-80191-11-3 in the Ames *Salmonella*/microsome plate test. Who Food Additives Series 31. ipcs. inchem. [Serial online: 1981] [Cited 2003 Nov 6]. Available from: <http://www.inchem.org/documents/jecfa/jecmono/v31je06.htm>.
 28. NI YC, HEFLICH RH, KADLUBAR FF, FU PP. Mutagenicity of nitrofurans in *Salmonella Typhimurium* TA98, TA98nr and TA98/1,8-DNP6. *Mutat Res* 1987; 192:15-22.
 29. CREBELLI R, CARERE A, FALCONE E, MACRI A. A study on the urinary and fecal excretion of furazolidone in rats by means of mutagenicity assays. *Ecotoxicol Environ Saf* 1982; 6: 448-456.
 30. CARERE A, CONTI L, CREBELLI R, MACRI A. Quantitative data on the urinary recovery of mutagenicity in furazolidone-treated rats. *Mutat Res* 1982; 97: 461-462.
 31. EBRINGER L, JURASEKA, KONICEK J, KONICKOVA M, LAHITOVA N, TRIBACUK S. Mutagenic action of nitrofurans on *Euglena gracilis* and *Mycobacterium phlei*. *Antimicrob Agents Chemother*. 197; 9: 682-689.
 32. MCCALLADRM, VOUTSINOS D. On the mutagenicity of nitrofurans. *Mutat Res* 1974;16:3-16.
 33. CHATTERJEE SN, BANERJEE SK, PAL AK, BASAK J. DNA damage, prophage induction and mutation by furazolidone. *Chem Biol Interac* 1982; 45:315-326.
 34. VOOGD CE, KRAMERS PGN, KNAA, AGAC, OUD JH, VAN WENT GF. Mutagenicity of feed additives. Poster session RIV, Bilthoven. Who Food Additives Series 31. IPCS. INCHEM. [Serial online: 1982] [Cited 2003 Nov 6]. Available from: <http://www.inchem.org/documents/jecfa/jecmono/v31je06.htm>.
 35. GAO N, NI YC, THORNTON-MANNING JR, FU PP, HEFLICH RH. Mutagenicity of nitrofurantoin and furazolidone in Chinese hamster ovary cell strains. *Mutat Res* 1989; 225:181-187.
 36. BRYANT DW, MCCALLA DR. Nitrofurans induced mutagenesis and error prone repair in *Escherichia coli*. *Chem Biol Interact* 1980; 31:151-166.
 37. OHTA T, NAKAMURA N, MORIYA M, SHIRASU Y, KADA T. The SOS-function-inducing activity of chemical mutagens in *Escherichia coli*. *Mutat Res* 1984; 131:101-109.
 38. BLIJLEVEN WGH, KORTSELIUS MJH, KRAMERS PGN. Mutagenicity testing of H-193, AF-2 and furazolidone in *Drosophila melanogaster*. *Mutat Res* 1977; 56:95-100.
 39. KRAMERS PG. Studies on the induction of sex-linked recessive lethal mutations in *Drosophila melanogaster* by nitroheterocyclic compounds. *Mutat Res* 1982; 101:209-236.
 40. SCHERES HME. Evaluation of the ability of furazolidone to induce chromosome aberrations in cultured peripheral human lymphocytes. Who Food Additives Series 31. IPCS. INCHEM. [Serial online:1991] [Cited 2003 Nov 6]. Available from: <http://www.inchem.org/documents/jecfa/jecmono/v31je06.htm>.
 41. COHEN MM, SAGI M. The effect of nitrofurans on mitosis, chromosome breakage and sister-chromatid exchange in human peripheral lymphocytes. *Mutat Res* 1979; 59:139-142.

42. TONOMURAA, SASAKMS. Chromosome aberrations and DNA repair synthesis in cultured human cells exposed to nitrofurans. *Jpn J Genet* 1973; 48:291-294.
43. QUEINNEC G, BABIL R, DARRE R, BERLAN, HM, ESPINASS J. Induction of abnormalities in chromosomes (of cattle and swine) by furazolidone or chloramphenicol. *Vet Bull* 1975; 46:330.
44. BABILE R, QUEINNEC G, BERLAND HM, DARRE R. Structure chromosomique des lymphocytes du porc et additifs alimentaires. *J Soc Biol* 1978; 172:546-553.
45. LU C, MCCALLA DR, BRYANT DW. Action of nitrofurans on *E. coli*. Mutation and induction and repair of daughter-strand gaps in DNA. *Mutat Res* 1979; 67:13-144.
46. PROBST GS, MCMAHON RE, HILL LE, THOMPSON CZ, EPP JK, NEAL SB. Chemically-induced unscheduled DNA synthesis in primary rat hepatocytes cultures: A comparison with bacterial mutagenicity using 218 compounds. *Environ Mutag* 1981; 3:11-32.
47. ENNINGA IC, WETERINGS PJJM. Micronucleus test in bone marrow cells of the mouse with furazolidone. WHO FOOD ADDITIVES SERIES 31. IPCS. INCHEM. [Serial online: 1990] [Cited 2003 Nov 6]. Available from: <http://www.inchem.org/documents/jecfa/jecmono/v31je06.htm>.
48. PAIK SG. Micronucleus induction in mouse bone marrow cells of some nitrofurans, 5-nitroimidazole and nitrothiazole derivatives used as trichomonacides in Korea. *Environ Mutat Carcinogens* 1985; 5:61-72.
49. FSANZ. Nitrofurans in Prawns. A toxicological Review and Risk Assessment. Technical Report Series. 2005 January M31.
50. SCHERES HME. Evaluation of the mutagenic activity of 3-amino-oxazolidinon-2 in the Ames *Salmonella*/microsome test. WHO Food Additives Series 31. IPCS. INCHEM. [Serial online: 1991] [Cited 2003 Nov 6]. Available from: <http://www.inchem.org/documents/jecfa/jecmono/v31je06.htm>.
51. AURO A, SUMANO H, OCAMPO L, BARRAGAN A. Evaluation of the carcinogenic effects of furazolidone and its metabolite in two fish species. *Pharmacogenomics J* 2004; 4: 24-8.
52. ALI BH. Pharmacological, Therapeutic and Toxicological Properties of Furazolidone: Some Recent Research. *Vet Res Commun* 1999; 23: 343-360.
53. SHITANDI AA, AILA O, OTTARO S, ALIONG O'L, MWAGI G *et al*. Effect of deep frying on furazolidone anticoccidial drug residues in liver and muscle tissues of chicken. *Afric J Food Sci* 2008; 2: 144-148.
54. AILA O, SHITANDI AA, MAHUNGU S, KUMAR-SHARMA H. Determination of the depletion of furazolidone residues in chicken tissues using a *Bacillus stearothermophilicus* test. *Food Control* 2009; 20:543-547.