Analysis of 3-Methyl-4-nitrophenol, A Major Metabolite of Fenitrothion, In Mice Urine Using HPLC

Abdrrahman Shemsu Surur[1*], Ahungena Alemayyehu[2] and Tarekegn Berhanu Esho[3]

[1]*Department of Pharmaceutical Chemistry, School of Pharmacy, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia*

[2]*Department of pharmaceutical chemistry and pharmacognosy, School of Pharmacy, Addis Ababa University, Addis Ababa, Ethiopia.*

[3]*Ethiopian Standard Agency, Addis Ababa, Ethiopia.*

ABSTRACT

Introduction: 3-methyl-4-nitrophenol is a major metabolite of fenitrothion and is considered as a potential risk to public health owning to its genotoxicity and carcinogenicity. **Objective:** To develop and validate an HPLC method for the quantification of 3-methyl-4-nitrophenol in urine. **Methods:** A sample of mice urine was collected following the administration of fenitrothion solution through oral gavage and subsequently, 3-methyl-4-nitrophenol was extracted using a simple liquid-liquid extraction method. The newly developed method uses acetonitrile: water 60:40 mobile phase at 270 nm with a flow rate of 1 mL/min HPLC condition. An optimization to a previously used extraction method was made by using ethyl acetate instead of acetonitrile to avoid extraction of water-soluble components of urine other than 3-methyl-4-nitrophenol. The developed method was validated using the ICH guideline for the validation of analytical procedures. Accordingly, the selectivity, linearity, limit of detection and quantification, accuracy, precision, extraction efficacy, robustness and stability of the method was determined. **Results:** All of the validation parameters showed that the method is valid for the determination of 3-methyl-4-nitrophenol in urine sample. Using this method it was possible to detect low concentration of 3-methyl-4-nitrophenol in urine samples down to 0.87 µg/mL. **Conclusion:** The sample was analyzed with this validated method and the respective concentration of the 3-methyl-4-nitrophenol was found to be 31.11 µg/mL. Accordingly, 10.37% of the total fenitrothion administered to mice was found metabolized into 3-methyl-4-nitrophenol. The HPLC retention time for 3-methyl-4-nitrophenol was 2.81 minutes.

Key words: Fenitrothion, 3-methyl-4-nitrophenol, HPLC, Metabolites, Method development, Validation.

INTRODUCTION

Fenitrothion (FEN) is an organophosphate insecticide used extensively throughout the world for the control of agricultural pests.[1,2] FEN is used, for example, to control the following coffee pest/diseases including berry borer, leaf miner, antesia bug, berry moth and mealy bug.[3,4] FEN is metabolized by hepatic cytochrome P450 to form fenitrooxon, which is further metabolized to dimethylphosphate and 3-methyl-4-nitrophenol (MNP) by paraoxonase 1. In another pathway, FEN is directly metabolized to MNP and dimethylthiophosphate by paraoxonase (Figure 1).[5]

MNP has been identified as the main degradation product of FEN in soil and the major metabolite of fenitrothion under both aerobic and anaerobic conditions.[6,7] Consequently, MNP has been measured as the specific exposure marker of FEN exposure. MNP is a potential risk to public health due to its genotoxicity and potential carcinogenicity according to organization for Economic Cooperation and Development.[2,8] MNP was shown to be more toxic to microorganisms than the parent compound.[9] MNP also found to inhibits ribonucleotide

***Corresponding address:**
Mr. Abdrrahman Shemsu Surur
Department of Pharmaceutical Chemistry,
School of Pharmacy, College of Medicine and Health Sciences,
University of Gondar, P.O. Box: 1181, Gondar, Ethiopia.
E-mail: lowerurexpect@gmail.com

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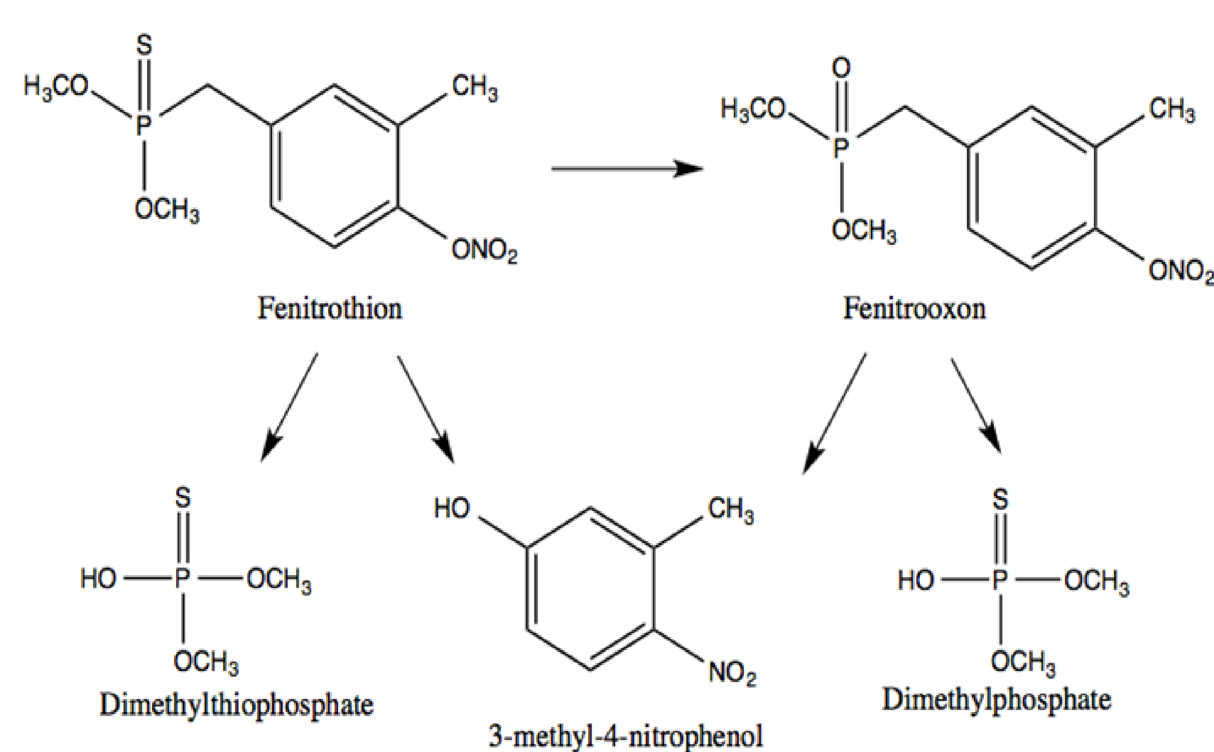

Figure 1: The major metabolites of fenitrothion

reductase, the enzyme solely responsible for the conversion of the four ribonucleotides to their corresponding deoxyribonucleotides required for DNA synthesis.[10] The strict control of the presence of MNP in food and water formed following the use of FEN is a matter of concern, as it has certain toxicological properties.

The main objective of this research was to develop an HPLC method coupled with LLE in order to determine MNP in mice urine. The use of HPLC for pesticide metabolite analysis, in contrast to GC-MS, is increasing due to the versatility and the very less preparative works needed.

Experimental

Chemicals and reagents

MNP standard (99.8% purity) was purchased from Nanjing Odyssey chemicals industry, China. All chemicals employed were analytical grade reagents. Acetonitrile and ethyl acetate gold for HPLC were purchased from Zaf pharmaceutical, which is a local representative of Carlo Erba, France. NaCl and Glacial acetic acid (Okhla industrials, New Delhi) and sodium acetate-3-hydrate (BDH laboratory supplies, United Kingdom) were made available at QC laboratory of East African pharmaceutical company, Ethiopia.

Instrumentation

SCHIMADZU HPLC system with DGU-20A3R degassing unit, LC-20AB column chromatograph, SIL-20A autosampler, CTO-20A column oven and SPD-20AV UV-Vis detector and dell core i3 computer with installed LC solution version 1.25 SP2 software was used. The separation column was packed with Kromasil 100-5 C18, 15 cm long, 5 μm film thickness and 4.6 mm internal diameter (akzonobel Bohus, Sweden).

Urine sample

A 20 mL of FEN solution in distilled water, which contains 0.34 mg of FEN, was prepared. Then water was added to make 30 mL of mixture. Eighteen mice each weighing between 30-40 g were fasted for 24 h but with access to water, and then 1 mL of FEN solution was administered by oral gavage. Six mice per a metabolic cage were kept after dosing. Urine samples were collected for 24 h after dosing. Another metabolic cage was allocated for the control group. Urine sample for control was collected in the same way except the mice were not dosed with FEN. One batch of urine sample was collected from the metabolic cages every week for three weeks. These three different batches were then mixed together to avoid inter-day variation. The urine samples, both treated and control, were then centrifuged at 10,000 rpm for 10 min at room temperature. The

supernatant were then collected and stored at -20°C.[11,12]

Sample extraction

0.5 mL of urine were pipetted into a 250 mL separatory funnel, to which 0.1 mL of sodium acetate buffer (pH 5.0) were added. This solution was incubated and mixed overnight at 37°C. Next, urine samples were transferred to a new separatory funnel, which were preliminarily heated at 180°C for 3 h, and about 100 mg of sodium chloride and 0.6 mL of ethyl acetate were added. The content was shaken in a horizontal direction for 20 min. The lower aqueous layer was discarded and the upper organic layer containing MNP was re-extracted twice with 0.6 mL of ethyl acetate. The extracts were evaporated using water bath. The residue was dissolved in 2 mL of acetonitrile and the sample was transferred to a sample vial.[5]

Sodium acetate buffer preparation

1.36 g of sodium acetate 3-hydrate was added to 100 mL of volumetric flask to which 0.6 mL of glacial acetic acid was added. Then water was added to produce 100 mL of solution. The pH of the solution was checked using pH meter and it was found to be 4.8. So sodium acetate 3-hydrate was added gradually until the pH becomes 5.[13]

HPLC analysis

SCHIMADZU HPLC coupled with UV detector was used for the determination of MNP using 60:40 acetonitrile: water was used as a mobile phase. The flow rate, injection volume and detection wavelength of the analysis were at 1.0 mL/min, 20 μL and 270 nm respectively.[14]

Method validation

The selectivity was evaluated by extracting and analyzing three different blank urine samples. The occurrence of possible interferences from endogenous substances was tested at the retention time of MNP.[15]

Linearity was evaluated by analyzing blank urine spiked with the standard solution at five different concentration levels: 5, 20, 35, 50 and 65 μg/mL. Extraction was performed and the extracted MNP was injected in triplicates. Linear correlation coefficient was used to estimate linearity.[15]

The limit of detection (LOD) and limit of quantification (LOQ) were used to determine the sensitivity of the method. LOD and LOQ were calculated based on the standard deviation of analyte response and slope of the calibration curve.[16,17]

Precision was investigated by calculating the relative standard deviation (RSD) at three urine concentrations corresponding to low (10 μg/mL), medium (35 μg/mL) and high concentrations (65 μg/mL). The intraday precision (repeatability) values were determined in three replicates at each concentration, and these replicates were processed independently with a two hours gap. The interday precision (reproducibility) values were determined across each concentration on three different days.[17,18]

Extraction recovery was calculated by comparing two experimental sets of data. In the first set, three blank urine samples were spiked before the extraction step with standard working solution at 10, 30 and 50 μg/mL final concentrations. In the second set, three blank urine samples were spiked after the extraction step, with MNP at the same final concentration of 10, 30 and 50 μg/mL. Recovery (%) was calculated as the ratio between the peak areas obtained from the two separate series of samples.[19,15]

Robustness was evaluated by modifying the following conditions: The mobile phase composition was changed from ACN: Water 60: 40 to 59: 41, the flow rate to 0.99 mL/min, the column temperature to 310 C and the injection volume to 19.9 μL. Three concentration levels (10, 30 and 50 μg/mL) were analyzed in duplicate. The compliance of retention time and the approximate concentrations was verified.[20]

To evaluate the freeze-thaw stability, three spiked urine samples at 10, 30 and 50 μg/mL were stored at -20°C for 7 days, and then thawed at room temperature. The temperature 20°C was selected since it is the temperature used to store the urine sample (biological samples). Samples were analyzed in three replicates and treated as described above. The samples stored at -20°C were compared with those of freshly prepared samples of the same concentrations. To be considered stable, the relative recovery (defined as the agreement between freeze–thawed and fresh samples) should be within 100 ± 15% or using RSD value of less than 2%.[18]

RESULTS AND DISCUSSION

Method Development

First, a solution of MNP in ethyl acetate was analyzed using acetonitrile (ACN): water 70:30 mobile phase, at 270 nm with a flow rate of 1 mL/min HPLC condition. The resulting peak was found to have a tailing problem (>2) and a retention time of 2 minutes (Figure 2). The mobile phase composition was changed to 50:50 since the decrease in the fraction of the organic solvent decreases the solvent

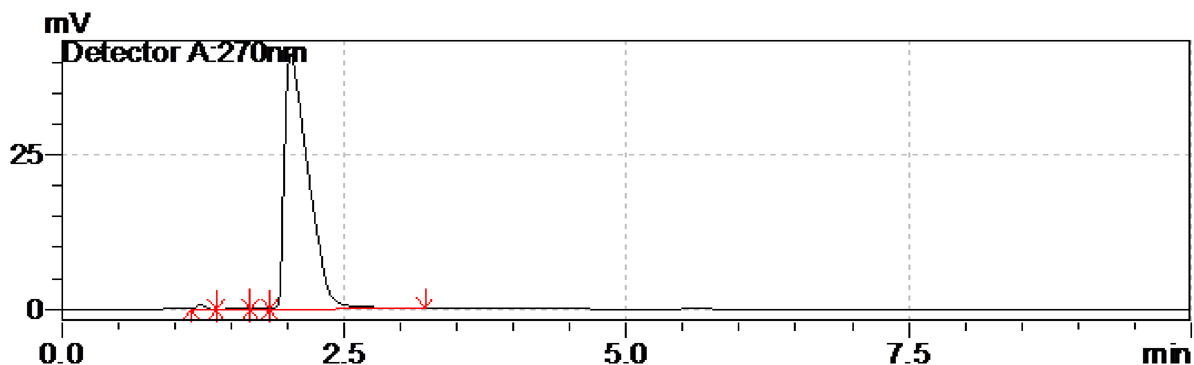

Figure 2: The chromatogram of MNP (35 μg/mL) in ethyl acetate using ACN: water 70:30 mobile phase

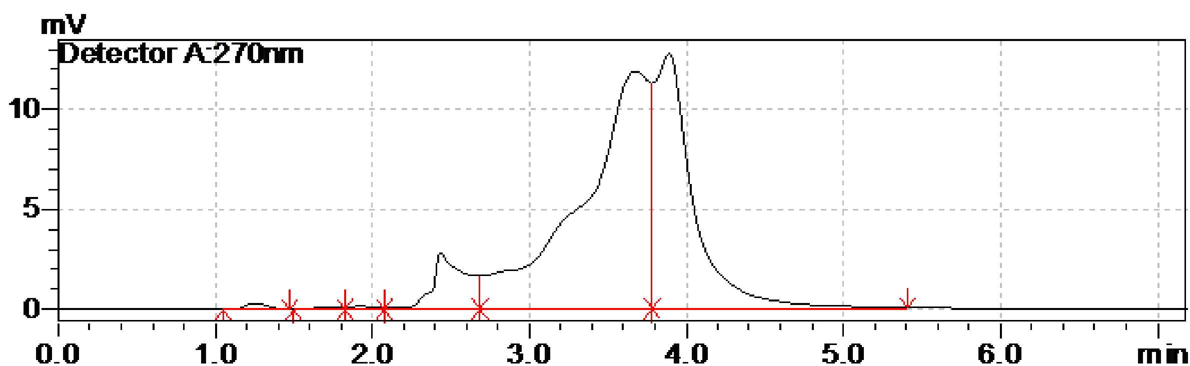

Figure 3: The chromatogram of MNP (35 μg/mL) in ethyl acetate using ACN: water 50:50 mobile phase

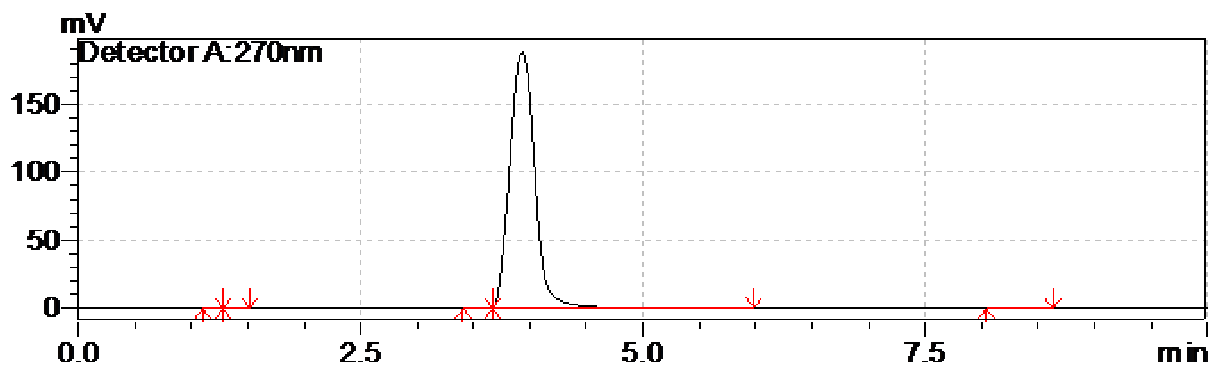

Figure 4: The chromatogram of MNP (0.5 mg/mL) in acetonitrile using ACN: water 50:50 mobile phase

strength and elution of the analyte, resulting in increased analyte retention.[21] However, the resulting peak was found to have a very long fronting (Figure 3).

A blank acetonitrile was analyzed at 270 nm and there was a very small absorption at 1.293 min and 3.976 min. A solution of MNP in acetonitrile was analyzed using ACN: water 50:50 mobile phase. A good peak was obtained at 3.93 min but the theoretical plate number was 1593.66 (Figure 4). When the mobile phase was changed to ACN: water 45:55, a better peak around 5 min was obtained with acceptable tailing factor 1.33 and theoretical plate 2794.18[22] (Figure 5). Finally the mobile phase was changed to ACN: water 60:40 and very good peak with a retention time of 2.783, tailing factor 1.323 and theoretical plate number of 2811 was obtained (Figure 6). Therefore, ACN: water 60:40 mobile phase composition and acetonitrile as a solvent was used for the method validation and analysis of the main sample.

A modification was made to a previously used extraction process.[11] A water bath was used to evaporate the extract instead of gentle nitrogen stream, which is not available at common pharmaceutical laboratories in developing

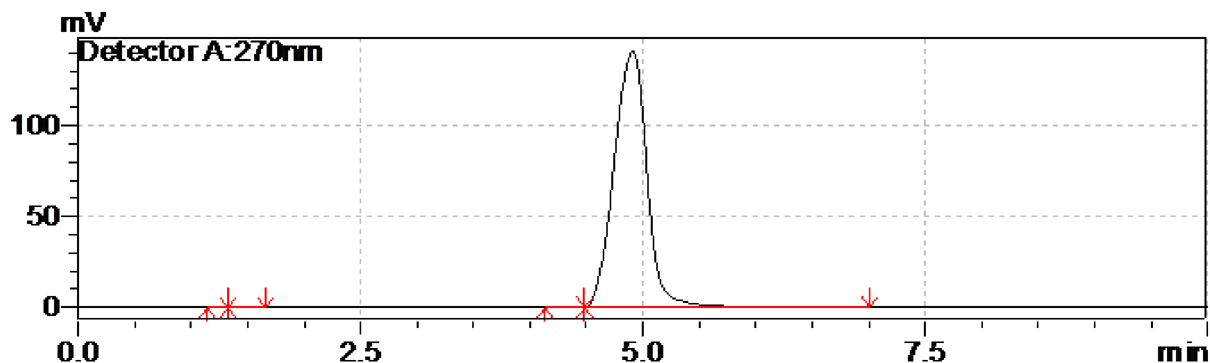

Figure 5: The chromatogram of MNP (0.5 mg/mL) in acetonitrile using ACN: water 45:55 mobile phase

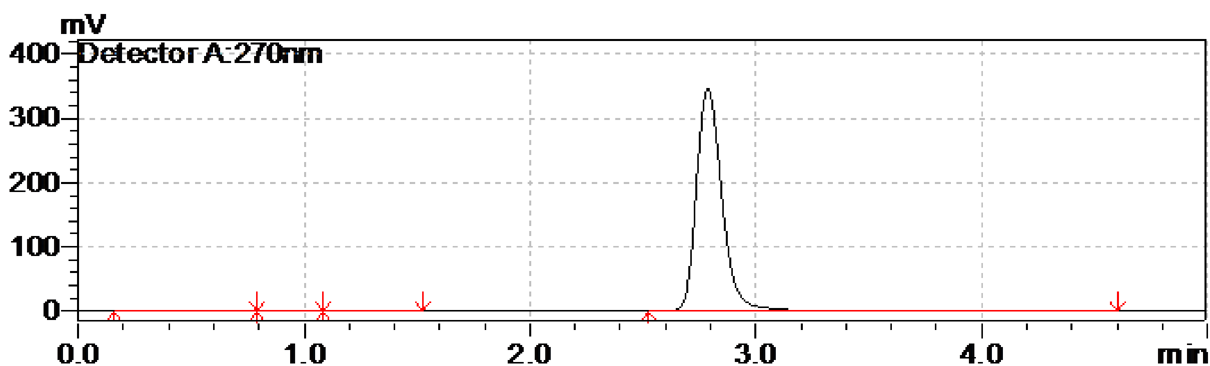

Figure 6: The chromatogram of MNP (0.5 mg/mL) in acetonitrile using ACN: water 60:40 mobile phase

countries. Ethyl acetate was used instead of acetonitrile to avoid the extraction of water-soluble components of the urine other than the target metabolites, which might create problems during HPLC analysis. Ethyl acetate seems to be sufficiently miscible with water to allow extraction of more polar pesticides. However, it is not completely miscible with water, which reduce the need for an extra partitioning step.[23]

After the extract was subjected to evaporation, the residue was dissolved in acetonitrile rather than ethyl acetate since acetonitrile gave a very good peak compared to ethyl acetate. This was probably due to the increase in the polarity of the component. Separatory funnels were used instead of test tubes since the separation of the organic phase from the aqueous phase was difficult with the use of test tubes. The re-extraction with ethyl acetate was done two times.

Method validation

According to ICH guidelines,[15] all validation parameters useful to evaluate the overall performance of an analytical method were investigated: including selectivity, linearity, sensitivity, precision, accuracy, recovery, robustness and stability.

Selectivity is concerned with the extent to which other substances interfere with the identification and quantification of the analyte of interest. Three blank mice urine samples were extracted and analyzed in a triplicate to check if there is any absorption between 2.79-2.83 min. The results ensured that there is no interfering signal either from the matrix or the extraction components at the retention time where the analyte of interest is expected to elute.

Methods are described as linear when there is a direct or proportional relationship between the method response and concentration of the analyte in the matrix over the range of concentrations. The calibration curve proved linear as demonstrated by correlation values of 0.999.

The LOD and LOQ of the method were calculated using the standard deviation and the slope of the calibration curve. The LOD value is calculated to be 0.87 units, which was the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The LOQ value is calculated to be 2.64 unit, which was the lowest concentration of analyte accurately detected and integrated by the instrument. The results suggest a good lower limit response of the method.[24]

Intraday precision is used to describe the variation of the method within the same day, while interday precision is for variation between different days. Intraday precision

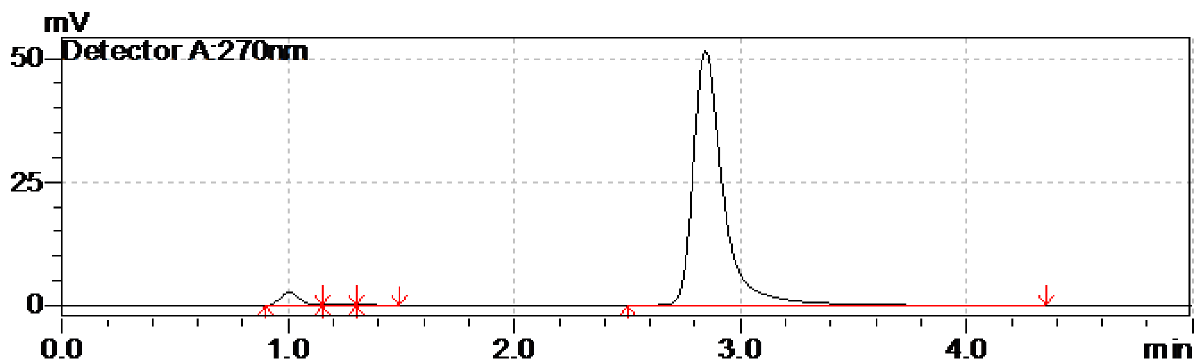

Figure 7: The chromatogram of mice urine sample collected following FEN administration

was checked using three different concentrations that cover low, middle and higher levels (5, 35 and 65 μg/mL) and all the concentration were injected in triplicates three times within the same day. The results indicated that the method is repeatable since the RSD is less than 2%.[25] The interday precision (reproducibility) studies also showed that the RSD value is less than 2.0%, which suggests the method is highly reproducible.[24,19]

Extraction recovery can be understood as the percentage of the metabolite originally in the biological specimen reaches the end of the procedure. For the extraction recovery, two experimental sets of data were used: Three blank urine samples were spiked before the extraction step and after the extraction for the first and second set respectively. Recovery (%) is used to express the extraction recovery for the two sets of data. The results showed that the extraction is efficient since the recovery value is greater than 80%.[14]

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. In this study the following changes were made and for eachparameters three concentrations (10, 30 and 50 μg/mL) were analyzed in triplicate; The mobile phase composition was changed from ACN: Water 60: 40 to 59: 41, the flow rate was changed from 1.00 to 0.99 mL/min, the column temperature was raised to 31^{0}C and the injection volume was reduced to 19 μL. The results showed that at the lower concentration level and medium concentration level those deliberate changes caused significant change in mean peak area only. Therefore, based on all the results, strictly following the HPLC procedures seems to be very important.

Stability was evaluated by comparing the mean peak area of freshly prepared sample with sample frozen at-20^{0}C for a week and then thawed at room temperature. Data to support the sample solution stability should be under normal laboratory conditions and for the duration of the test procedure. This was the reason for using -20°C for 7 days, which was the temperature condition and the period the urine sample was stored before analyzed.[22] The results indicated that MNP had no significant degradation under the condition previously described.

Analysis of the sample

The proposed method was applied to the determination of the amount of MNP in mice urine sample collected following the administration of FEN. The urine sample was extracted and injected in triplicate. An average peak with retention time of 2.84 and mean peak area of 477831 was obtained (Figure 7). The respective concentration was calculated using the equation f (x)=15288X + 2135 of the calibration curve and found to be 31.11 μg/mL, 10.37% of the total FEN administered to mice. This showed that significant amount of the administered FEN is metabolized to MNP.

CONCLUSION

This research showed that LLE coupled with HPLC is a successful technique for the quantification of MNP in mice urine. Good precision, accuracy and extraction recoveries are obtained, with a detection limit of 0.87 μg/ml. However, the method was not robust enough since the RSD value was higher than the acceptable limit.

The HPLC retention time under the experimental condition described for MNP was 2.81 minutes and the measured concentration of MNP from mice urine was 31.11 μg/ml after the administration of 0.3 mL of fenitrothion to the mice. Accordingly, 10.37% of the administered FEN was metabolized to MNP.

The analytical approach developed in this paper could easily be applied to the biological monitoring of human exposure to MNP due to a similar metabolism of FEN among mammals.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

MNP : 3-methyl-4-nitrophenol
FEN : Fenitrothion
HPLC : High performance liquid chromatography
RSD : Relative standard deviation
ACN : Acetonitrile
LOD : Limit of detection
LOQ : Limit of quantification
LLE : Liquid-liquid extraction

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