BIOMARKERS, 1998, VOL. 3, NO. 1, 81–87

SHORT COMMUNICATION

α–Fluoro-β-alanine in urine of workers occupationally exposed to 5-fluorouracil in a 5-fluorouracil-producing factory

R. P. BOS†*, B. F. J. WEISSENBERGER‡ and R. B. M. ANZION†

† Department of Toxicology, University of Nijmegen, 6500 HB Nijmegen, The Netherlands

‡ F. Hoffmann-LaRoche AG, Occupational Health Service, 4070 Basel, Switzerland

Received 9 May 1997, revised form accepted 1 September 1997

Because of possible harmful health effects increased attention is being paid to the occupational exposure to cytostatic drugs of workers in hospitals and industry. In this study a biomarker for exposure to 5-fluorouracil (5FU) based on GC–MSMS was applied to study the occupational exposure of four workers in a pharmaceutical factory producing 5FU. The four workers all excreted α-fluoro-β-alanine (FBAL), a metabolite of 5FU, via the urine (range excretion rates: 0–88·9 μg per 8h). This is in accordance with the presence of 5FU and/or its precursor ethoxyfluorouracil (EFU) in stationary and personal air samples as well as in wipe samples taken from the floor at the workplace.

Keywords: biological monitoring, 5-fluorouracil, occupational exposure, antineoplastic agents, cytostatic drugs.

Abbreviations: 5FU, 5-fluorouracil; FBAL, α-fluoro-β-alanine; EFU, ethoxyfluorouracil; cv, coefficient of variation.

Introduction

5-Fluorouracil (5FU) is one of the most frequently used antineoplastic drugs. In addition to the therapeutic effects a number of side effects are known. The first acute signs of toxicity in patients are leucopenia and stomatitis (IARC 1981). Other side effects are diarrhoea, nausea, vomiting, thrombocytopenia and a decrease in erythrocyte precursors in the marrow. Alopecia, dermatitis, hyperpigmentation and photosensitivity have also been reported. With respect to possible chronic toxicity of 5FU, the results of an animal carcinogenicity study were considered to be inadequate (IARC 1981). Genotoxicity studies in bacteria were inconclusive (IARC 1987a, b). Neither chromosome aberrations nor sister chromatid exchanges were induced in peripheral blood lymphocytes of patients treated with 5FU. 5FU induced micronuclei but not specific locus mutations in mice *in vivo*. It induced aneuploidy, chromosomal aberrations, and sister chromatid exchanges in cultured Chinese hamster cells. It did not induce sex-linked recessive lethal mutations in *Drosophila*, but caused genetic crossing over in fungi. The compound was found to be embryotoxic in animals.

The biotransformation of 5FU in rodents as well as in man has been described (IARC 1981). In a patient study it was found that 80 % of the dose is quickly excreted in urine as α-fluoro-β-alanine (FBAL) (Bernadou 1985).

* To whom correspondence should be addressed.

1354-750X/98 $12.00

Several groups of workers, such as hospital workers involved in the preparation and administration and of 5FU workers in industry involved in the production and compounding of 5FU, are exposed to this drug occupationally. However, it is hardly known to what extent these workers are really exposed. We have recently developed some methods to quantify occupational exposure to cytostatic drugs among which are cyclophosphamide, methotrexate and 5FU (Sessink *et al.* 1992, 1993, 1994a, b, Bos and Sessink 1997). In a previous study we have investigated the exposure to 5FU in a pharmaceutical plant where workers were involved in drug compounding and drug production (Sessink *et al.* 1994b). To establish the uptake of 5FU, urine samples were analysed for the presence of α-fluoro-β-alanine (FBAL) using GC–MS. A concentration of 50 μg FBAL per litre was found in the urine of one employee. This level was just above the detection limit. In the meantime we have further optimized the method for detection of this biomarker of exposure. In the present paper we describe the application of this more sensitive method in a study of the occupational exposure of factory workers involved in the production of 5FU.

Methods

A small occupational health and hygiene study was performed on three successive days during a 6-week 5-fluorouracil (5FU) campaign. During this period environmental as well as biological monitoring strategies were applied.

Production process

In the plant 5FU is produced by hydrolysis of ethoxyfluorouracil (EFU). For that purpose EFU is placed in a kettle and hydrolysed. After hydrolysis the product is crystallized and centrifuged two times. Next, the product is dried. Finally, about 420 kg of 5FU is produced and divided into barrels.

Some EFU dust may spread around during loading and unloading of the kettle and during the taking of samples. To prevent further distribution the EFU is moistened.

Probably the greatest possibility for the contamination of the work atmosphere and exposure of the workers is located in a cabin close to the production room. Here the employees are involved in the administration and handling of the product(s) and removal of the barrels. EFU is loaded via the inlet of the forementioned kettle. Although it appeared that in the case of unloading the kettle a lot of visible dust was produced, during loading the workers (worker 2) were more protected and probably subjected to a lower exposure.

Workers

The foreman (worker 1) was probably not highly exposed. He was in the production room for only 1–2 h per shift. Worker 2 was involved in filling the kettle with EFU. He wore a coverall (Arjo-Sic AG, Basel Swiss; Cat Nr.07565), an airstream helmet (L. Giffard SA, Plaintel Swiss; Cat Nr.5005-V) and latex gloves (Weita, Arlesheim Swiss; Cat Nr.26-253.100). No personal air samples were collected from this person. Worker 3, involved in handling the barrels (preparation and transport), was partly protected. This person sometimes wore P2 or P3 masks (Boleij *et al.* 1995) and sometimes a coverall without a cap. He always wore gloves.

Worker 4 was working in the laboratory and involved in the process and quality control, handling the sifting machine.

Wipe samples

Wipe samples were taken from two places on the floor of the above mentioned cabin (A) and from in front of the cabin (B) in order to detect contamination of the work area with 5FU. Samples were taken after filling of the kettle and after sifting in the laboratory.

The sample area (100 cm^2) was wiped with a round filter (12·7 mm i.d., Schleicher and Schuell) which had been prewetted with 100 μl deioinized water. Samples were stored and finally extracted in

10 ml glass stoppered tubes. Before the extraction procedure 1 ml of the internal standard solution was added. Next the filters were treated ultrasonically for 10 min and shaken for 15 s. After filtration the clear samples were transferred to HPLC vials. Thymine was used as internal standard (4 μg ml^{-1}).

Air samples

Stationary air samples were taken in the cabin 1·5 m above the location where wipe samples A were taken and in front of the inlet at a height of 1·7 m above the location where wipe samples B were collected. An SKC-Pump 224-PCXR-7 was used. Total airborne particulate matter was collected on glassfibre filters (Millipore, AP 4003705) having a diameter (and cone) of 37 mm. It was estimated that a sampling time of 4 h with a suction flow of 3·5 l min^{-1} resulted in an air sample corresponding to a volume of 0·84 m^3.

Personal air samples were taken close to workers 1, 3 and 4 with the same type of pump and same type of filters as used for the stationary sampling. In this case the cone was only 4 mm at an equal suction rate of 3·5 l min^{-1}. Workers 1 and 3, who wore the pump for 4 h, and worker 4, who wore the pump for 8 h sampled 0·84 and 1·68 m^3 of air, respectively.

Stationary and personal air samples were taken at the same time for 4 or 8 h (see above). Filling of the kettle with EFU took place in the middle of this period and lasted about 1·5 h.

The filters were removed from the sampling head and transferred to 10 ml glass stoppered tubes. The heads were rinsed with 1 ml of water which was added to the tube. One ml of internal standard solution was added and extraction took place as described above. A calibration solution was prepared by mixing 1 ml of a 5FU solution (5 μg ml^{-1}) with 1 ml of the internal standard solution (see Wipe Samples).

For stationary and personal air samples the detection limit of 5FU was 0·08 μg per filter.

Urine samples

Urine samples were collected from three of the workers (1, 2 and 3) on four successive days. Worker 4 collected urine samples for only 3 days. Urine samples were collected after starting work on the first day (late shift) over periods of 8 h. The urine samples were frozen at –20 °C until assayed.

Chemicals

5FU (purity > 99 %) used as a reference in the laboratory was obtained from Janssen Chimica (Beerse, Belgium) and FBAL (purity > 99 %) was purchased from Interchim (Montluçon, France). *S*-Ethyltrifluorothioacetate (purity > 99 %) was obtained from Sigma Chemical Company (St Louis, MO). All other chemicals used were of the highest purity obtainable.

HPLC analysis of 5FU

HPLC analyses were performed on an HP1090 instrument equipped with a Lichrospher 100-RP18 column (5 μm, 250 × 4 mm, Merck) at room temperature. Solvent A (100 % methanol) and solvent B (5 % methanol in purified water) were used for gradient elution at a total flow of 1 ml min^{-1}. For 7 min, 100 % B was applied to the column; this was followed by a fast gradient to 100 % A in 3 min and an isocratic region of 100 % A for 8 min. Next, the initial condition of 100 % B was restored. Aliquots of 10 μl were injected and detection took place at 265 nm. The retention time of 5FU is 4·5 min. The limit of detection was 0·04 μg 5FU ml^{-1}.

GC-MSMS analysis of FBAL in urine

Sample preparation and derivatization

The sample preparation procedure was performed as described by Sessink *et al.* (1994b) with slight modifications. One ml of urine and 100 μl of a 5 mol L^{-1} sodium hydroxide solution were combined in tubes with screw caps. After addition of 150 μl of *S*-ethyltrifluorothioacetate, the tubes were tightly closed and shaken for 5 h at room temperature (Schallenberg *et al.* 1955). One hundred microlitres of concentrated hydrochloric acid were added and the solution was mixed. The samples were extracted twice with 3 ml of ethylacetate. The combined ethylacetate layers were dried under nitrogen at 50 °C. After addition of 0·5 ml of freshly prepared acidified *n*-butyl alcohol (250 μl of acetyl chloride in 5 ml of *n*-butyl alcohol) and subsequent mixing, the tubes were closed for butylation for 1 h at 90 °C. The

samples were cooled down to room temperature and dried under nitrogen at 40 °C. Next, 750 μl of toluene was added and vials were filled with sample and stored at –20 °C until analysis.

GC and MS conditions

Analyses were performed on a Varian Saturn 4D GCMSMS controlled by a Compaq Prolinea 4/50 personal computer. The oncolumn injection mode was used (SPI: Septum equipped Temperature Programmable Injector). Aliquots (1 μl) were injected on a 30 m DB-5 column (J & W Scientific, Folsom, CA, USA) with 0·25 mm internal diameter and 0·25 μm film thickness by means of an 8200 CX autosampler (Varian). The column was connected to a deactivated fused silica retention gap (Varian, Houten, The Netherlands) with a length of 5 m and an internal diameter of 0·53 μm. The initial injection temperature was 110 °C. After 1 min the temperature was increased by 90 °C min^{-1} to 280 °C. After 3 min the temperature was decreased to the initial temperature by cooling with compressed air.

The initial oven temperature was 110 °C. After 1 min the temperature was increased by 7·5 °C min^{-1} to 140 °C, next by 20 °C min^{-1} to 290 °C, where it remained constant for 7 min. Helium was used as carrier gas (column inlet pressure 14·5 psi). The interface temperature was 290 °C. The manifold (ion-trap) temperature was 230 °C. The retention time of derivatized FBAL was 5·5 min.

The ion-trap MS was operated in the MSMS-mode. Ion fragment m/z 186, one of the intense fragments after initial EI-ionization, was trapped as the parent-ion. In the second stage an excitation amplitude of 27 V (non-resonant) and an excitation-RF of 300 DAC were applied to produce daughter-ions. Identification was carried out by the combination of retention time and MSMS-spectrum. Daughter-ion m/z 158 was used for quantification. The peak area of derivatized FBAL was calculated and compared with freshly prepared calibration curves of FBAL in reference urine in a range of 12·7–381 μg l^{-1}. Compared with the earlier described EI-based method by Sessink *et al.* (1994b) we were able to decrease the detection limit 10-fold to approximately 6 μg l^{-1} (signal: noise ratio > 3).

Urine samples of five laboratory technicians of the Department of Toxicology (Nijmegen) not involved in this occupational hygiene study were analysed for the presence of FBAL. No FBAL was found. During this study 10 FBAL standards were analysed in duplicate (range: 12·7–381 μg l^{-1}). The overall cv was 6·9 % (coefficient of variation = $\Sigma\%D/n\sqrt{2}$).

Results and discussion

In personal air samples (table 1) as well as stationary air samples (table 2) the presence of 5FU was confirmed. It appeared that the concentration of 5FU in the cabin was higher than in front of it. Worker 3 showed higher concentrations of 5FU in the inhalation zone than workers 1 and 4. The difference is not significant, possibly because of the low number of observations. The spread of 5FU was also detected on the floor. At several locations the floor was contaminated (table 3). The values found for contamination of the floor were highest in the production room and lowest in the laboratory. Only in the laboratory did the usual cleaning procedure seem partly successful.

From the results of the analyses of the wipe samples it can be concluded that the floor of the production room was highly contaminated, followed by the area in front of the cabin, in the cabin, and in the laboratory, respectively. The release of 5FU was confirmed by the presence of 5FU on the filters of the stationary and personal air samples. Whereas the stationary air samples show higher values in the cabin compared with in front of the cabin, the wipe samples show no difference. The data indicate that the workers were exposed to 5FU aerosols and suggest that inhalation is an important exposure route. In addition, skin contamination and subsequent uptake through the skin cannot be excluded.

Table 4 shows the urinary excretion rates of FBAL in the four workers. Worker 3 showed the highest excretion rates (mean 136·4 μg FBAL per 24 h), worker 4 had the lowest excretion rates (mean 11·3 μg FBAL per 24 h). The values measured in worker 3 were significantly higher than workers 1, 2 and 4 (Mann Whitney test, $p < 0{\cdot}0001$, $p = 0{\cdot}0002$ and $p = 0{\cdot}011$, respectively). It is interesting to note that the higher excretion rates of FBAL in worker 3 are accompanied by

Table 1. Personal air sampling of 5FU (μg m^{-3}).

Worker	Sampling time (h)	Day 1	Day 2	Day 3	Day 4	Mean
1	4		2·6	0·8	0·9	1·4
3	4		6·3	5·4	2·4	4·7
4	8	0·6	1·1	0·7		0·8

Table 2. Stationary air sampling of 5FU (μg m^{-3}).

Location	Sampling time (h)	Day 2	Day 3	Day 4	Mean
In the cabin	4	0·33	0·87	0·21	0·47
In front of cabin	4	0·27	0·38	0·19	0·28

higher values of 5FU measured during personal air sampling of this worker compared with the other workers. A remarkable correlation was observed between the excretion rates at the successive excretion periods of worker 1 and worker 3 ($r = 0{\cdot}69$, $p = 0{\cdot}02$).

It is clearly shown that worker 2, wearing a coverall, an airstream helmet and latex gloves, has a lower urinary excretion rate for FBAL than worker 3, who was involved in handling the barrels and had no clearly defined protection. This demonstrates the efficacy of the measures taken to prevent worker 2 from uptake of 5FU by inhalation and via the skin. Whether the workers had their highest exposure by inhalation or via skin contact cannot be concluded from these results.

The correlation between the excretion rates of worker 1 and worker 3 can be explained by a comparable exposure pattern of these two persons. The absence of a correlation with worker 2 might be explained by the protective clothing and other hygiene measures taken by this person. The absence of a correlation with worker 4 may be explained by the distance of this person from the place where workers 1 and 3 are exposed.

We wondered whether a conclusion could be drawn from the urinary excretion rates of FBAL with respect to an optimal sample collection period. For this reason we have compared the excretion rates of the workers measured over the periods 06–14 h, 14–22 h and 22–06 h. In the Wilcoxon signed rank test we found a significant difference between the excretion rates of the four workers when we compared their excretion rates at 14–22 h with those of 06–14 h (Wilcoxon paired non-parametric, two-tailed $p = 0{\cdot}027$), with the highest excretion rates between 14 and 22 h. In the same test we found a significant difference between the excretion rates between 22–06 and 06–14 h, with the highest excretion rates between 22 and 06 h, considering the data of workers 1, 3 and 4 (Wilcoxon paired non-parametric, two-tailed $p = 0{\cdot}016$). When also the values of worker 2 are involved, the difference between the time periods disappeared. Since workers 1, 2 and 3 worked from 14 to 22 h (late shift) and worker 4 from 8 to 17 h (day shift), this means a relatively short half-life of 5FU in this occupational exposure situation. This is in agreement with the relatively short physical half-life of about 2 h as determined in pharmacokinetic studies in patients (Shani *et al.* 1982).

Table 3. Contamination of the floor with 5FU (μg per 100 cm^2).

Location	Day 1	Day 2	Day 3	Day 4	Mean
Before cleaning					
A		6·9	5·1	3·7	5·2
B		11·1	4·7	5·8	7·2
C		25·6	7·3	5·9	13·0
L	0·95	0·75	0·55		0·75
After cleaning					
A		13·0	6·2	5·5	8·2
B		20·8	11·8	6·2	12·9
C		23·3	18·0	21·9	21·1
L	0·38	0·45	0·27		0·37

A: In the cabin.
B: In front of the cabin.
C: In the production room.
L: In the laboratory.

Table 4. Urinary excretion rate of FBAL in workers of a 5FU-producing plant (μg per 8 h).

Day	Time of shift (h)	Worker 1	Worker 2	Worker 3	Worker 4[a]
1	06–14	ns[b]	ns	ns	nd[c]
1	14–22	10·1	10·5	49·7	3·3
1	22–06	14·5	nd	88·9	nd
2	06–14	5·0	22·9	30·3	3·5
2	14–22	8·1	6·4	59·8	5·0
2	22–06	7·3	11·2	44·3	4·1
3	06–14	6·4	8·9	29·3	13·7
3	14–22	9·0	26·6	48·0	nd
3	22–06	24·4	2·1	68·4	15·7
4	06–14	4·5	16·3	15·7	ns
4	14–22	12·3	16·9	38·0	ns
4	22–06	nd	6·7	28·6	ns
5	06–14	6·7	16·7	44·4	ns
Mean urinary excretion rates of FBAL during this period (μg FBAL per 24 h):		27·1	36·3	136·4	11·3

[a] Real sample time was 1 h later for this worker.
[b] ns: No urine sampled.
[c] nd: Concentration lower than 5·7 μg l^{-1} urine.

The results of this study show that in this factory the work environment is contaminated with 5FU and that the workers are exposed to 5FU and/or EFU during the manufacturing process. Considering the chemistry of EFU (easily hydrolysable to 5FU) it can be expected that exposure to this compound will also result in the excretion of FBAL. The applied methods seem useful for the detection of occupational exposure in this situation. In particular, the method for the measurement of FBAL in urine as a consequence of the uptake of 5FU (and EFU) suggests that this biomarker of exposure is well suited for use in situations in which exposure to 5FU can be expected. In our previous paper concerning occupational exposure to 5FU we found FBAL in the urine of only one worker (Sessink *et al.* 1994b). In that study about 50 μg FBAL was detected in a urine sample collected between 10 and 13·75 h after beginning the weighing of 5FU. In

the meantime the limit of detection has been lowered from approximately 50 ng ml^{-1} urine to 6 ng ml^{-1} urine. The improvement is attributed to the use of MSMS. The parent ion mass $m/z = 186$ is stored in the ion-trap and further dissociated in a so-called daughter ion with $m/z = 158$ which is very specific for FBAL. Of the 45 urine samples that were analysed in this study, only five samples had FBAL levels below the detection limit. Our previous method would have given between 35 and 40 samples below the detection limit. In this case we only should have been able to measure excretion of FBAL in worker 3. Further validation of this method in situations with occupational exposure to 5FU (in hospitals, pharmaceutical plants, etc.) is needed to gain further insight into the applicability of the method.

Acknowledgements

The authors thank Dr S. Rönninger and J. Geiger for performing and interpreting the HPLC analysis and D. P. Geissberger who was involved in the occupational hygiene study.

References

BERNADOU, J., ARMAND, J. P., LOPEZ, A., MALET-MARTINO, M. C. and MARTINO, R. 1985, Complete urinary excretion profile of 5-fluorouracil during a six-day chemotherapeutic schedule, as resolved by ^{19}F Nuclear Magnetic Resonance. *Clinical Chemistry*, **31**, 846–848.

BOLEIJ, J. S. M., BURINGH, E., HEEDERIK, D. and KROMHOUT, H. 1995, *Occupational Hygiene of Chemical and Biological Agents* (Amsterdam, Tokyo: Elsevier), pp. 242–253.

BOS, R. P. and SESSINK, P. J. M. 1997, Biomonitoring of occupational exposure to cytostatic anticancer drugs. *Reviews on Environmental Health*, **12**, 43–58.

IARC 1981, *Monographs on the evaluation of carcinogenic risks to humans. Vol. 26. Some antineoplastic and immunosuppressive agents* (Lyon, France: International Agency for Research on Cancer).

IARC 1987a, *Monographs on the evaluation of carcinogenic risks to humans. Suppl. 6. Genetic and related effects: an updating of selected IARC monographs from volumes 1 to 42* (Lyon, France: International Agency for Research on Cancer).

IARC 1987b, *Monographs on the evaluation of carcinogenic risks to humans. Suppl. 7. Overall evaluations of carcinogenicity: an updating of IARC monographs volumes 1 to 42* (Lyon, France: International Agency for Research on Cancer).

SCHALLENBERG, E. E. and CALVIN, M. 1955, Ethyl thiotrifluoracetate as an acetylating agent with particular reference to peptide synthesis. *Journal of the American Chemical Society*, **77**, 2779.

SESSINK, P. J. M., BOER, K. A., SCHEEFHALS, A. P. H., ANZION, R. B. M. and BOS, R. P. 1992, Occupational exposure to antineoplastic agents at several departments in a hospital. Environmental contamination and excretion of cyclophosphamide and ifosfamide in urine of exposed workers. *International Archives of Occupational and Environmental Health*, **64**, 105–112.

SESSINK, P. J. M., SCHOLTES, M. M., ANZION, R. B. M. and BOS, R. P. 1993, Determination of cyclophosphamide in urine by gas chromatography–mass spectrometry. *Journal of Chromatography*, **616**, 333–337.

SESSINK, P. J. M., FRIEMÈL, N. S. S., ANZION, R. B. M. and BOS, R. P. 1994a, Biological and environmental monitoring of occupational exposure of pharmaceutical plant workers to methotrexate. *International Archives of Occupational and Environmental Health*, **65**, 401–403.

SESSINK, P. J. M., TIMMERMANS, J. L., ANZION, R. B. M. and BOS, R. P. 1994b, Assessment of occupational exposure of pharmaceutical plant workers to 5-fluorouracil. Determination of α-fluoro-β-alanine in urine. *Journal of Occupational Medicine*, **36**, 79–83.

SHANI, J., YOUNG, D., SCHLESINGER, T., SIEMSEN, J. K., CHLEBOWSKI, R. T., BATEMAN, J. R. and WOLF, W. 1982, Dosimetry and preliminary human studies of ^{18}F-5-fluorouracil. *International Journal of Nuclear and Medical Biology*, **9**, 25–35.