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Biological monitoring of occupational exposure to 5-fluorouracil: Urinary α-fluoro-β-alanine assay by high performance liquid chromatography tandem mass spectrometry in health care personnel☆

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ABSTRACT

A new sensitive and specific HPLC–MS/MS method for the determination of α-fluoro-β-alanine (FBAL), the main metabolite of the antineoplastic drug 5-fluorouracil (5-FU), in urine for the biological monitoring survey of health care workers exposed to 5-FU is described. This procedure is characterized by a pre-column FBAL derivatization by 2,4-dinitrofluorobenzene followed by solid phase extraction sample clean-up. The chromatographic separation was achieved by hydrophilic interaction chromatography (HILIC) on a ZIC HILIC column (Sequant) and the quantification was performed by tandem mass spectrometry. The method offers high sensitivity with a quantification limit of 1 μg/l, which is an improvement on those previously reported. The within- and between-day precisions were less than 13% and 15% respectively at the LOQ and no significant relative matrix effect was observed for FBAL. The validated method was applied to the biological monitoring of occupational exposure to 5-FU in a French hospital. Pre- and post-shift urine samples were collected from 19 workers in a hospital pharmacy and an oncology ward over a period of 5 days. On a total of 121 analysed samples, measurable amounts of FBAL were detected in up to 29%, the concentrations range from LOQ to 22.7 μg/l, yielding evidence of occupational exposure to 5-FU. Such data are scarce and represent a step forward in assessing the occupational health risks associated with handling antineoplastic drugs.

1. Introduction

Antineoplastic drugs (ANDs) are widely used in cancer therapy because they can inhibit growth by disrupting cell division and killing actively growing cells. These agents can also cause health effects among the health care workers who work with them. Adverse effects to exposed workers such as hair loss, skin rashes [1–3] and reproductive effects [4–7] have been reported. Furthermore, some ANDs have a carcinogenic, teratogenic or mutagenic potential [8].

Occupational exposure to antineoplastic drugs can occur in hospital pharmacies and in hospitals where ANDs are prepared or administered to patients, mainly through inhalation and skin contact [9–11]. The health risks related to handling antineoplastic drugs have therefore become a major concern for occupational medicine in hospitals. Several guidelines for the safe handling of antineoplastic have been developed by professional associations and health agencies [12–15].

However, to evaluate the occupational health risks associated with handling these drugs, reliable quantitative data on exposure levels are needed. Biological monitoring can be used advantageously to assess personal exposure as it takes into account all the exposure routes. Since it is unrealistic to measure all antineoplastic drugs, the most frequently used compounds, including cyclophosphamide, ifosfamide, methotrexate and 5-fluorouracil (5-FU), have generally been used as occupational exposure markers.

Among these compounds 5-FU has indeed emerged as a promising biomarker. It is widely employed in hospitals and is highly metabolized in humans. After administration, 5-FU undergoes two metabolic pathways. The drug is anabolized to the nucleotide level to exert its cytotoxic effects against tumour cells while the catabolic pathway leads to the reduction of the heteroaromatic ring to 5,6-dihydro5-fluorouracil and further ring opening produces α-fluoro-β-alanine (FBAL), an unnatural amino acid [16,17], as shown in Fig. 1. Approximately 60–90% of 5-FU administered dose were excreted in urine within 24 h, mainly as FBAL [16,18].

However, only a few analytical methods have been published to quantify FBAL in a biological matrix [19–22], and only two of them involved biological monitoring of occupational exposure to

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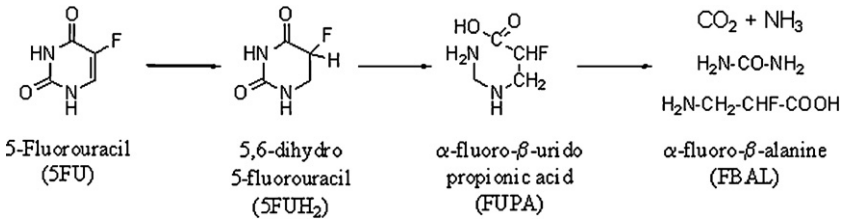

Fig. 1. Proposed catabolic pathway for 5-FU [16].

antineoplastic drugs [19,22] using gas chromatography–mass spectrometry.

In this paper, a new validated analytical method based on high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS), for the determination of urinary FBAL, is described. This procedure is characterized by FBAL derivatization followed by solid phase extraction (SPE) sample clean-up and hydrophilic interaction chromatography (HILIC). This method allows quantification of FBAL at μg/l level and is therefore appropriate for monitoring occupational exposure to 5-fluorouracil.

2. Materials and method

2.1. Chemicals and reagents

α-Fluoro-β-alanine (FBAL) was supplied by Interchim (Montluçon, France) with a purity >99%. 2,4-Dinitrofluorobenzene (DNFB) was purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). β-Alanine-d4, used as internal standard, was obtained from CDN isotopes (USA) with a purity >98%. Sodium borate and phosphoric acid were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Acetonitrile (LC–MS grade) was purchased from Merck (Darmstadt, Germany). Ammonium formate (LC–MS grade) was obtained from Fluka (Saint-Quentin Fallavier, France). Ultrapure water was produced by a Direct Q system from Millipore (Saint-Quentin en Yvelines, France).

2.2. Standard solutions

Stock solutions of FBAL and internal standard were prepared in pure water at 1 g/l and stored at −20 °C. Intermediate solutions at 10 mg/l were prepared in pure water and stored at −20 °C. Appropriate serial dilutions of intermediate solutions with a pool of human urine from members of our laboratory staff were prepared just before use for calibration in the range 1–100 μg/l.

2.3. Sample preparation

To a 1 ml urine sample, 100 μl of internal standard (β-alanine-d4, 2 mg/l) was added, followed by 500 μl of a 10 mM sodium borate solution and 400 μl of the derivatizing reagent (DNFB at 10 g/l in acetonitrile). The mixture was vortexed and heated at 65 °C for 30 min. After heating, 50 μl of 50 mM phosphoric acid was added. The reaction mixture was then cooled down at room temperature and extracted by solid phase extraction (SPE).

The automated SPE procedure was performed on RapidTrace extractor (Caliper Life Sciences, Tremblay-en-France, France) using 60 mg/3 ml Oasis HLB cartridges (Waters, Saint-Quentin en Yvelines) as follows. The reagent lines were primed by purging them with methanol, 15 mM phosphoric acid, methanol/15 mM phosphoric acid (10/90) and acetonitrile successively. After conditioning the SPE cartridge with methanol (2 ml) and 15 mM phosphoric acid (2 ml), 1 ml of the reaction mixture was loaded. Thereafter, the cartridge was washed with 2 ml of methanol/15 mM phosphoric acid (10/90) and the analytes were eluted with 2 ml of acetonitrile. The eluate was evaporated to dryness under a stream of nitrogen and the residue was redissolved in 2 ml of mobile phase then injected into the HPLC–MS/MS.

2.4. Chromatographic conditions

The samples were analysed on a HPLC–MS/MS system. This consisted of a liquid chromatography system using two Varian Prostar 210 pumps, a Prostar 410 autosampler and a Varian 1200 L Triple quadrupole mass spectrometer system with an ESI interface. Five different columns were evaluated: Polaris C_{18} column (150 mm × 2.1 mm, 3 μm) from Varian (Les Ulis, France), Xterra MS C_{18} column (100 mm × 2.1 mm, 3.5 μm) from Waters (Saint-Quentin-en-Yvelines, France), Atlantis dC_{18} column (150 mm × 2.1 mm, 3 μm) from Waters, Atlantis HILIC column (150 mm × 2.1 mm, 3 μm) from Waters and Sequant ZIC-HILIC column (100 mm × 2.1 mm, 5 μm) from AIT (Houilles, France). Data acquisition and quantification were performed with a Varian 6.9 MS Workstation.

The mobile phase used was a gradient of 25 mM ammonium formate solution in pure water and acetonitrile. The starting eluent, an ammonium formate–acetonitrile mixture (5:95, v/v), was applied for the first 2 min. The proportion of ammonium formate was then increased to 10% over a period of 3 min. The mobile phase was then immediately adjusted to its initial composition and elution was continued for 10 min in order to re-equilibrate the column. The flow rate of the mobile phase was 0.2 ml/min and the injection volume 5 μl. The column was thermostated at +50 °C. The retention times were 5.2 and 3.8 min respectively for FBAL derivative and internal standard derivative.

The Triple quadrupole mass spectrometer operated in negative mode with the following parameters: ESI needle voltage, −5000 V; API drying gas (nitrogen), 250 °C, 21 psi; API nebulizing gas (air), 40 psi; detector voltage, 1285 V; collision cell gas pressure (argon), 1.56 mTorr. The transition precursor ion/product ion was m/z 258/182 for β-alanine-d4 derivative and 272/182 for FBAL derivative.

2.5. Validation study

The validation criteria of the method were assessed in pools of spiked urine samples. Linear regression analysis was used to construct calibration curves. Calculation of the concentrations was performed via the peak area ratios from the FBAL derivative to the internal standard derivative. Within-day and between-day precisions and accuracy were evaluated by determining FBAL in three quality control (QC) samples prepared at nominal urine concentrations of 1, 5, 20 μg/l in six replicates on 3 different days. The precision of the method at each QC concentration was expressed as a coefficient of variation (CV) by calculating the standard deviation as a percentage of the mean calculated concentration, while the accuracy of the procedure was determined by expressing the mean calculated concentration as a percentage of the added concentration.

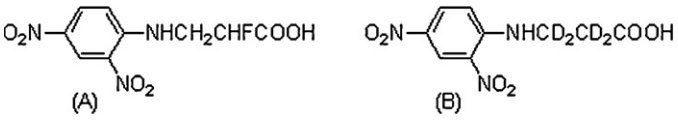

Fig. 2. Chemical structures of DNB–FBAL (A) and DNB–β-alanine-d4 (B).

The limit of quantification (LOQ) was determined by analysing nine replicates of blank urine spiked with internal standard. LOQ is defined as ten times the standard deviation of the blank at the same retention time as the FBAL derivative.

To assess the reliability of the overall method, relative matrix effect was evaluated by determining the precision of standard line slopes (expressed as CV%) in four different urine lots, as described by Matuszewski [23].

2.6. Field study

A total of 19 subjects from two workplaces of a French hospital gave their consent to take part in this investigation. The workplaces were a hospital pharmacy and an oncology ward. Urine samples were collected before and after work shifts from pharmacy technicians, nurses and auxiliary nurses. Five consecutive days were considered in this study and a total of 121 urine samples (64 pre- and 57 post-shift samples) were collected and stored at −20 °C before analysis.

Most of the workers handling antineoplastic drugs used gloves and masks. ANDs were prepared in a normal room environment equipped with a positive air pressure isolator.

3. Results and discussion

3.1. FBAL derivatization and HPLC–MS/MS

To quantify FBAL with high sensitivity, a specific and selective method able to distinguish FBAL from the other small endogeneous molecules of similar molecular weight in the urine was needed. HPLC–MS/MS coupled with pre-column derivatization was therefore chosen in order to improve the detection level. Sanger's reagent, 2,4-dinitrofluorobenzene (DNFB), was chosen as the reagent for FBAL derivatization. DNFB reacted readily with FBAL and internal standard β-alanine-d4 at an elevated temperature by forming dinitrobenzene (DNB) derivatives, as shown in Fig. 2. The derivative solution remained stable for at least 1 week at 5 °C in darkness [24,25], and derivatization at 65 °C for 30 min resulted in total conversion. Furthermore, this reaction proceeded in an aqueous solution, which is highly desirable when analysing a biological sample.

Both derivatives were ionized in the ESI source and gave corresponding $[M-H]^-$ ions. CID fragmentation of $[DNB–FBAL–H]^-$ m/z 272 and $[DNB–β-alanine-d4–H]^-$ m/z 258 produced one predominant ion, namely m/z 182.

Three different C_{18} reversed-phase columns were first evaluated for the separation of FBAL and β-alanine-d4 derivatives, using a mixture of water–acetonitrile as mobile phase. The two compounds were eluted near the void volume on Polaris column. The retention increased on X-Terra column, however broad and tailing peaks were observed. The derivatives were resolved enough on Atlantis dC18 column but mobile phase with more than 80% water was needed, which is unsuited to HPLC–MS/MS. Hydrophilic interaction liquid chromatography (HILIC) was then used to achieve chromatographic separation of the FBAL and β-alanine-d4 derivatives, due to their highly polar properties. HILIC offers an attractive alternative to reversed-phase liquid chromatography (RPLC) insofar as compounds that have little or no retention on RPLC columns generally experienced strong retention on the HILIC column. The HILIC technique bears similarities with traditional normal phase liquid chromatography, the important difference being that HILIC employs semi-aqueous mobiles phases. HILIC is thus well suited to mass spectrometry since typical eluents consist of 40–97% acetonitrile in water or volatile buffer [26,27]. Two HILIC phases, underivatized silica Atlantis HILIC and sulfobetaine zwitterionic ZIC HILIC, were investigated. ZIC HILIC column was found to be least affected by changes in buffer pH and needed less time to reach equilibrium than Atlantis HILIC column. Therefore, ZIC HILIC was used for subsequent analyses. Optimization of the organic content, buffer concentration and pH showed that gradient separation with a mobile phase of 25 mM ammonium formate–acetonitrile mixture produced good chromatography. Typical HILIC chromatograms, obtained from the analysis of human blank urine and a urine sample from an auxiliary nurse, are showed in Fig. 3.

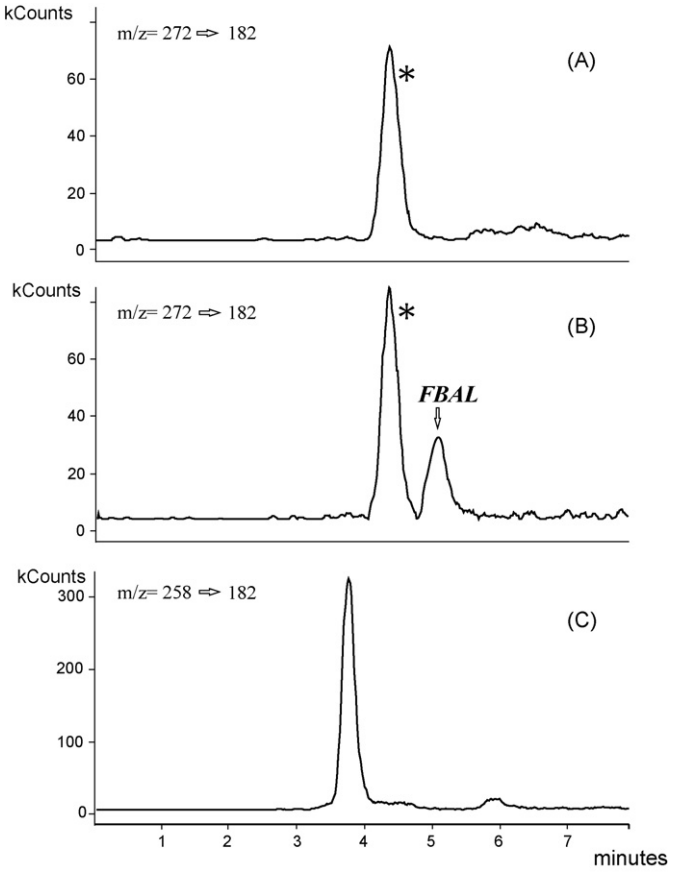

Fig. 3. MRM chromatograms for DNB–FBAL from blank human urine (A), auxiliary nurse exposed to 5-FU urine, FBAL concentration: 2.30 μg/l (B) and the internal standard (C). *Urine impurity.

3.2. Validation study

The calibration curves were linear in the range 0–100 μg/l with correlation coefficients higher than 0.992.

The within-day and between-day precision and accuracy of the method were assessed by analysing six replicates of three quality control samples (1, 5, 20 μg/l). The results are reported in Table 1. The within-day precision varied from 5.6% to 12.5% at the lower concentration while between-day precision ranged from 6.5% to 14.5%. The method was shown to be accurate, with intra-day accuracy always higher than 91%.

Overall method reliability was investigated by determining relative matrix effects in four different urine lots, covering a broad

Table 1
Within-day and between-day precision and accuracy of the urinary FBAL determination method.

Nominal concentration μg/l urine, $n = 6$	Within-day precision CV (%)	Between-day precision CV (%), $n = 3$ days	Accuracy % of nominal concentration
1	12.5	14.5	91
5	6.2	8.0	95
20	5.6	6.5	97

Table 2
FBAL concentrations in the urine samples of pharmacy technicians, nurses and auxiliary nurses.

Parameter	Pharmacy technicians	Nurses	Auxiliary nurses
Study participants (n)	6	5	8
No. of urine samples	52	26	43
No. of participants with positive sample	5	2	7
No. of samples ≥LOQ	15	4	16
Percentage of positive samples (%)	28.8	15.4	37.2
Concentration range (μg/l)	1.17–6.06	1.27–22.7	1.00–9.85

range of urines, i.e. low, normal and high in creatinine. The precision of the standard line slopes, expressed as CV (%), did not exceed 6.5%, indicating that the method, given within- and between-day precisions reported above, can be considered reliable and free from relative matrix effect liability.

The urinary limit of quantification of the assay was found to be 1 μg/l. At this concentration, the within-day precision was 12.5% and the accuracy 91%. This LOQ is the lowest ever published so far. Previous methods published by Sessink et al. [19] and Rubino et al. [22] relied on biological monitoring of occupational exposure to 5-FU and yielded sensitivities of 60 and 20 μg/l respectively. Our method is therefore more sensitive than previously published analytical procedures, e. g. using gas chromatography coupled with mass spectrometry.

3.3. Field study

Nineteen workers, 6 involved in preparation and 13 in administration, exposed to 5-FU in a French hospital were monitored over a 5 days. FBAL was found once or several times in 14 of the 19 subjects, mainly in the post-shift samples. In the hospital pharmacy, FBAL was detected in 5 of the 6 pharmacy technicians, the concentrations ranging from 1.17 to 6.06 μg/l. In the oncological ward, we determined the presence of FBAL in the urine of 9 of the 13 nurses and auxiliary nurses with concentration ranging from 1.00 to 22.7 μg/l. The pharmacy technicians had 15 positive results, the nurses 4, and the auxiliary nurses 16 positive urine samples (Table 2).

Despite standard safety precautions, FBAL was thus detected in urine samples from 74% of the participants and in 29% of the overall samples collected, proving 5-FU contamination among these workers.

In the most recent previous study where biological monitoring of workers exposed to 5-FU was performed, Rubino et al. [22] found 3 positive results among the 64 samples, with FBAL concentrations of 20, 30 and 1150 μg/l, so fewer positive samples than in our study in proportion. One obvious explanation for this difference is that our analytical method is very more sensitive, all the positive results in our study (except one sample at 22.7 μg/l) being below the limit of detection of 20 μg/l published by Rubino et al. [22].

Finding FBAL in urine despite standard safety precautions when handling ANDs proves that there are other sources of contamination. These sources are probably places where workers do not consider themselves to be contaminated and touch without taking precautions; dermal uptake has been demonstrated to be the major route of exposure. Moreover, ANDs contamination of different locations in hospitals has been highlighted by wipe samples [28–32].

A further relevant finding of our study was that auxiliary nurses appeared more contaminated than nurses in the oncology ward (Table 2). The percentage of positive urine samples was about 37% for auxiliary nurses and 15% for nurses. Oncology auxiliary nurses performed nursing tasks like handling patients' urine, washing patients and removing their bed sheets. They usually wore vinyl gloves, known to be permeable to ANDs. They reported however that gloves were not worn regularly when washing patients or removing their bed sheets. Furthermore, auxiliary nurses were generally unaware of all the potential sources of contamination during their tasks. These findings suggest that some workers such as auxiliary nurses may be inadequately informed about the health risks related to handling ANDs and about occupational exposure. Adequate education and training must be provided and dispensed to all workers, and safety guidelines and protective measures should be drawn up and made available.

4. Conclusion

To our knowledge, this is the first published method based on HILIC–MS/MS for the determination of FBAL in the urine of occupationally exposed workers. This validated analytical method is reliable, very sensitive (at least 20 fold more sensitive than previous methods using other analytical techniques) and is able to detect urinary FBAL at μg/l level. Our method has been applied to biological monitoring of occupational exposure to 5-FU. The data obtained show 5-FU uptake by a high percentage of pharmacy technicians, nurses and auxiliary nurses despite standard safety precautions. Our study also indicates that the auxiliary nurses were more contaminated than the nurses in this hospital. Training and adequate protective measures are therefore needed for these workers. On account of its sensitivity, our method is thus suitable for biomonitoring occupational exposure to 5-FU in different posts including pharmacy technicians, nurses, auxiliary nurses; cleaning personnel can also be followed up. Moreover, this method should cover not only the exposure to 5-FU but also exposure of 5-FU generating prodrugs like capecitabine. These results represent a step forward in assessing the occupational health risks associated with handling antineoplastic drugs. Indeed, such data on 5-FU exposure are scarce, probably due to the lack of sensitivity of the previously reported methods. This investigation will continue with an assessment of the current situation in various workplaces. It will hopefully turn out to be a valuable tool for monitoring the effectiveness of the measures implemented and help occupational hygienists to keep exposure as low as practicable.

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