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2,4-Difluoroaniline and 4-fluoroaniline exposure: monitoring by methaemoglobin and urine analyses

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Summary. Two possible methods for monitoring exposure to 2,4-difluoroaniline and 4-fluoroaniline have been investigated: measurement of methaemoglobin content in blood and measurement of urinary metabolites. Experiments using rats dosed by the oral route as a model system show that measurement of methaemoglobin content provides a very rapid and simple monitoring method, but is not very sensitive. Measurement of the *ortho*-hydroxy metabolites of the two compounds, as their benzoxazole derivatives, provides a much more sensitive, but complicated technique. Details of both methods are presented.

Key words: 2,4-difluoroaniline – 4-fluoroaniline – Exposure monitoring – Rats – Urine – Blood – Metabolites

Introduction

2,4-Difluoroaniline (DFA) and 4-fluoroaniline (PFA) are two of a number of halogenated anilines, manufactured by the Halex process, for use as fine chemical intermediates. A method of monitoring possible exposure of plant workers to the two compounds (Ii and Iii in Fig. 1) was required. Two different approaches were investigated: analysis of urinary metabolites and determination of methaemoglobin content in blood.

Methaemoglobin is a form of haemoglobin where the iron atoms at the centre of the haems have been reduced to the ferric form (Smith 1969). Methaemoglobin is unable to combine reversibly with oxygen, and possesses a different ultraviolet absorption spectrum from that of haemoglobin. A wide range of anilines can induce methaemoglobinaemia in both animals and man (Greenberg and Lester 1947; McLean et al. 1969; Kiese and Weger 1969; Bright and Marrs 1982; Harvey and Keitt 1983), but compounds with a substituent at the 4-position have been shown to be particularly potent inducers (Kiese 1963).

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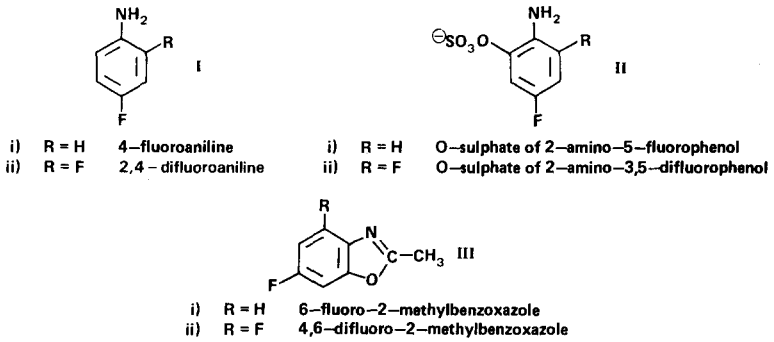

Fig. 1. Structures of compounds

Prior to this study, nothing was known of the metabolism of DFA or PFA in animals or man. However, substituted anilines are readily metabolised after ingestion to give *ortho* and *para* hydroxy derivatives (Parke and Williams 1956; Grunow et al. 1970). When the *para* position is blocked by an inert substituent (such as a halide), *ortho* hydroxylation is often the predominant metabolic pathway (Baldwin and Hutson 1980). The hydroxyanilines, often conjugated as sulphates or glucuronides, are readily excreted in the urine.

The methaemoglobin formation and the excretion of *ortho*-hydroxysulphate metabolites by rats after dosing with 2,4-difluoroaniline and 4-fluoroaniline were investigated to determine if they could form the basis of possible methods for monitoring human exposure to the compounds.

Materials and methods

Materials

2,4-Difluoroaniline (DFA) was supplied by Shell Chemicals UK. Stanlow and 4-fluoroaniline (PFA) was supplied by the Aldrich Chemical Company, Dorset. 4-Chloroaniline (the positive control substance), potassium ferricyanide and potassium cyanide were obtained from BDH, Dorset and Nonidet P40 was supplied by Shell Chemicals UK. The test compounds and the positive control were shown to be pure, and stable in corn oil for five days at room temperature, by ^{19}F and ^{1}H nuclear magnetic resonance spectroscopy (nmr). Corn oil was "Mazola" brand obtained locally. Other chemicals were of general reagent grade.

Animals

All animals used in these studies were male rats of the Wistar strain from the colony at Sittingbourne Research Centre, Shell Research Ltd.

Determination of methaemoglobin content

The doses shown in Table 1 were prepared no more than 24 h before administration. The tolerance on the doses was ± 2%. Each animal (average weight 243 g, range 220 to 267 g) received its dose in 2 ml per kg of corn oil. For each dose level, the dosing schedule was as follows: groups of six rats were starved overnight and the following morning four animals were given the test compound (i.e. either 2,4-difluoroaniline or 4-fluoroaniline), the fifth animal received 4-chloroaniline (the positive control) and the sixth animal received corn oil (the negative control). Animals were allowed free access to food 1 h after dosing. Water was given *ad libitum*.

Table 1. Doses of the test compounds administered orally to rats for both metabolite and methaemoglobinaemia production

2,4-Difluoroaniline			4-Fluoroaniline			4-Chloroaniline (positive control)		
mg ml^{-1}	mg kg^{-1}	mmol kg^{-1}	mg ml^{-1}	mg kg^{-1}	mmol kg^{-1}	mg ml^{-1}	mg kg^{-1}	mmol kg^{-1}
64	128	1.0	56	112	1.0	63	127	1.0
32	64	0.5	28	56	0.5			
16	32	0.25	14	28	0.25			
8	16	0.13	7	14	0.13			

Blood samples (approximately 250 μl) were taken by tail vein bleeding 1 h before, and at 1, 2, 4, 6, 8 and 24 h after dosing. Methaemoglobin was determined by a modification of the method of Evelyn and Malloy (1939). Whole blood (100 μl) was lysed in 5 ml of a mixture of freshly prepared phosphate buffer (0.1 *M* pH 6.8) and Nonidet P40 (10 ml l^{-1}) (2:3, v/v). The methaemoglobin content was calculated from the absorbance at 630 nm before and after the addition of potassium cyanide solution (20 μl, 5%) to about 1 ml of the lysed blood in a UV cuvette. The results were expressed as a percentage of the total blood pigment, using samples of the same lysed blood (ca 1 ml) to which potassium ferricyanide solution (20 μl, 5%) had been added 2 to 5 min earlier.

Stability of methaemoglobin

In order to determine how long the methaemoglobin persist in blood after sampling, a sample of tail vein blood was taken from a rat 30 min after it had been dosed with 2,4-difluoroaniline (128 mg per kg of body weight). Analysis of the sample at 30-min intervals showed that the percentage of methaemoglobin decreased with time, indicating that at room temperature, the methaemoglobin was not stable. The half-life for the disappearance was about 70 min; hence all samples were analysed as soon as they had been collected.

Isolation and identification of the metabolites from rats dosed with 4-fluoroaniline (PFA) or 2,4-difluoroaniline (DFA)

Twenty rats with an average weight of 255 g (range 216 to 309 g) were housed in all-glass metabolism cages (2 animals per cage) designed for the separate collection of urine and faeces. The animals were starved overnight and on the following morning ten of the animals were given 4-fluoroaniline, and another ten given 2,4-difluoroaniline. Doses (128 mg DFA (1 mmol) kg^{-1} body weight or 112 mg PFA (1 mmol) kg^{-1} body weight) were prepared no more than 24 h before administration and stored at 4°C ± 2C° until use. Each animal received its dose in 2 ml kg^{-1} of corn oil. After dosing, animals were allowed free access to food; water was given *ad libitum*. Urines from each group of rats were collected for the period 0 to 24 h, mixed and stored at −20°C until required. Urines were subsequently filtered through Whatman No. 1 filter paper under suction. Cetylpyridinium bromide (1.2 g) was added as an aqueous solution and the products were allowed to precipitate slowly overnight at 4°C. The white solids were separated by centrifugation, washed several times with distilled water and recrystallised from acetone.

The structures of the urinary metabolites of PFA and DFA, isolated as the cetylpyridinium salts, were confirmed, using nuclear magnetic resonance spectroscopy (nmr) (Bruker WM 360 nmr spectrometer) and elemental analysis, to be the 0-sulphates of 2-amino-5-fluorophenol and 2-amino-3,5-difluorophenol respectively (IIi and IIii in Fig. 1). The ^1H spectrum of the purified metabolite of 4-fluoroaniline, as its cetylpyridinium salt, was consistent only with an aniline which had been metabolised by *ortho* substitution; the protons showed characteristic coupling constants for a 1, 2, 4 substituted aromatic system and the coupling constants expected for

protons *ortho* and *meta* to a fluorine substituent. The chemical shifts of the protons were also consistent with an *ortho*-phenolsulphate. The precise details of the region of the spectrum where the aromatic protons occurred were: (C_3D_6O) δ 6.54 (1H, td, $J_{H\text{-}F}$8, $J_{H\text{-}H}$8,2), 6.64 (1H, m, $J_{H\text{-}F}$8, $J_{H\text{-}H}$7), 7.05 (1H, dd, $J_{H\text{-}F}$8, $J_{H\text{-}H}$2). The corresponding ^{1}H spectrum for the metabolite of 2,4-difluoroaniline was also only consistent with a 2, 4, 6 substituted aniline, the proton to proton coupling of 2.9 Hz being typical of protons *meta* to each other. The chemical shifts and proton to fluorine couplings were also consistent with an *ortho*-phenolsulphate. The precise details of the region of the spectrum where the aromatic protons occurred were: (C_3D_6O) δ 6.59 (1H, m, $J_{H\text{-}F}$11.0, 8.9, $J_{H\text{-}H}$2.9) 6.93 (1H, m, $J_{H\text{-}F}$10.3, 2.0, $J_{H\text{-}H}$2.9).

Confirmatory evidence that the *ortho*-substituted metabolites of PFA and DFA contained a sulphate moiety was provided by elemental analysis of the metabolites, in the cetylpyridinium form. (Formula $C_{27}H_{43}N_2SO_4F$ found C 63.9%, H 8.7%, N 5.4%, S 6.7%; calculated C 63.5%, H 8.4%, N 5.5%, S 6.3% – Formula $C_{27}H_{42}N_2SO_4F_2$ found C 62.3%, H 8.2%, N 5.4%, S 6.2%; calculated C 61.4%, H 8.0%, N 5.3%, S 6.1%).) The metabolites were then converted into their respective benzoxazoles (Fig. 1) using a mixture of acetic acid and acetic anhydride with BF_3 as catalyst, and the benzoxazole structures were confirmed by mass spectrometry (see section on 'Analytical procedure').

Metabolite excretion following dosing rats with 4-fluoroaniline and 2,4-difluoroaniline

Rats with an average weight of 250 g (range 218 to 281 g) were individually housed in all-glass metabolism cages. Groups of four rats were dosed, as previously outlined in 'Determination of methaemoglobin content', except that no animals were given the controls.

Urine was collected before dosing, i.e. pre-exposure and for the periods 0 to 24 h and 24 to 48 h after dosing. For each dose the individual urine volumes were recorded; the urine samples were mixed and stored at −20°C until required. The urine samples were analysed for the respective conjugated aminophenol metabolites of PFA and DFA. The analytical method involves conversion of these metabolites in a single step reaction into the respective benzoxazoles (Fig. 1), using a mixture of acetic acid and acetic anhydride with boron trifluoride as catalyst (see 'Analytical procedure').

Analytical procedure

Urine samples (5 ml) were evaporated to dryness on a water bath and then refluxed for 1 h with 45% (v/v) acetic acid in acetic anhydride (10 ml) and BF_3 etherate (0.7 ml). The product was transferred to a 250-ml separatory funnel with water (20 ml), and 12% (w/v) aqueous sodium carbonate (140 ml) was added to neutralise acidic material and decompose the acetic anhydride. After standing for 15 min, a 50:50 diethyl ether/hexane mixture (50 ml) was added, and the mixture was shaken. The aqueous layer was discarded and the organic layer was washed with further portions of 12% (w/v) sodium carbonate (50 ml) and saturated sodium chloride solution (25 ml). The organic extract was then cleaned up using an oxidative procedure. A saturated solution of potassium permanganate in acetone (3 ml) was added, followed by the addition, with shaking, of a saturated ferrous sulphate solution in 2% (v/v) aqueous sulphuric acid after a period of 15 min. The organic layer was run off and diluted to 50 ml with hexane.

The hexane extract was analysed for the respective benzoxazoles using a Hewlett Packard gas-liquid chromatograph, model 5730A, fitted with a nitrogen thermionic detector (GC-NTD). Suitable glass columns were found to be: (i) 1.4 m × 2 mm id containing 3% (m/m) of Dexsil 300 GC on 100/120 mesh Gas Chrom Q support, operated at 85°C for 6-fluoro-2-methylbenzoxazole and (ii) 1.8 m × 2 mm id containing 3% (m/m) of Dexsil 400 GC on 100/120 mesh Gas Chrom Q support, operated at 95°C for 4,6-difluoro-2-methylbenzoxazole. Instrumental conditions were set in accordance with the manufacturer's recommendations. The results from GC-NTD for the 0 to 24 h urine samples were confirmed by mass spectrometric detection by monitoring a molecular ion of mass characteristic of the benzoxazoles (GC-SIM). For this confirmatory work a Finnigan 4000 gas chromatography/mass spectrometer with both electron impact and chemical ionization (CH_4 reagent) was used.

The analytical procedure was also applied to control human urine samples and pre-exposure rat urine samples (to establish the limit of detection of the method) and to the control samples to which known quantities of the cetylpyridinium salt of the 0-sulphate of 2-amino-5-fluorophenol or 2-amino-3,5-difluorophenol had been added for calibration purposes. External standard solutions of the respective benzoxazoles were not used for calibration, rather a range of benzoxazole standards were prepared in situ by the quantitative conversion of added quantities of the cetylpyridinium salt of the 0-sulphates (1 to 10 μg ml^{-1}, expressed as percent fluoroaniline) in control urine. The in-situ standards were prepared by refluxing these metabolite standards, after removal of water, with the acetic/acetic anhydride reagent mixture, with BF_3 as catalyst as described previously.

Results and discussion

Methaemoglobinaemia

Methaemoglobin content in rats was determined after oral dosing of 1.0, 0.5, 0.25 and 0.13 mmol kg^{-1} of 2,4-difluoroaniline and 4-fluoroaniline. For each compound, at each dose level groups of four male rats were treated, together with a single male rat given the positive control (1.0 mmol kg^{-1} of 4-chloroaniline), and a single negative control given the dosing vehicle (corn oil). Even at equimolar doses, neither of the test compounds was as potent an inducer of methaemoglobin as was the positive control compound.

The average methaemoglobin content in each group at each dose is given in Figs. 2 and 3. At all doses the maximum methaemoglobinaemia occurs rapidly,

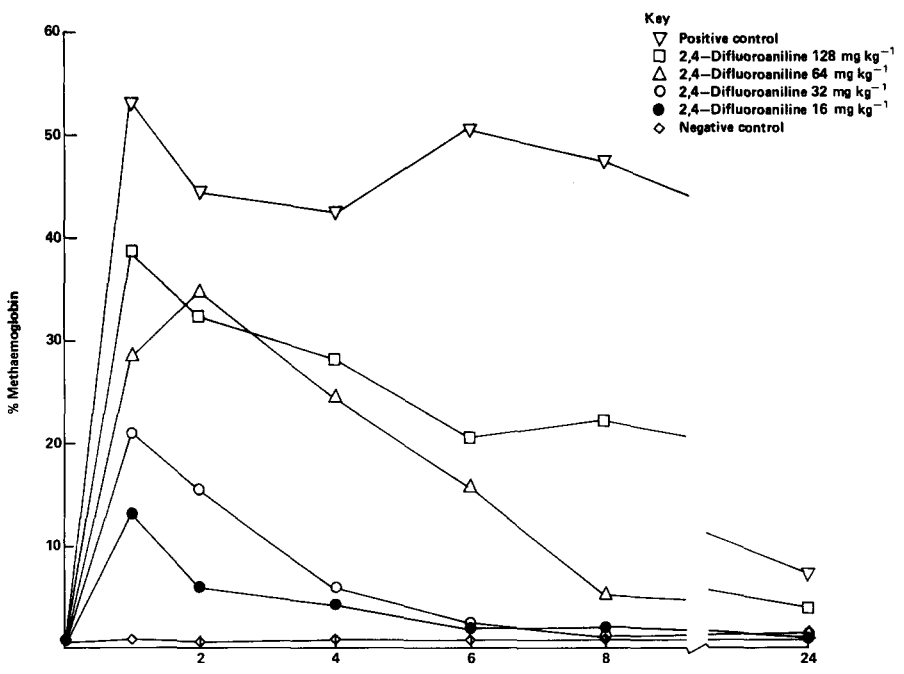

Fig. 2. Variation of methaemoglobinaemia with time and dose after single oral doses of 2,4-difluoroaniline to rats

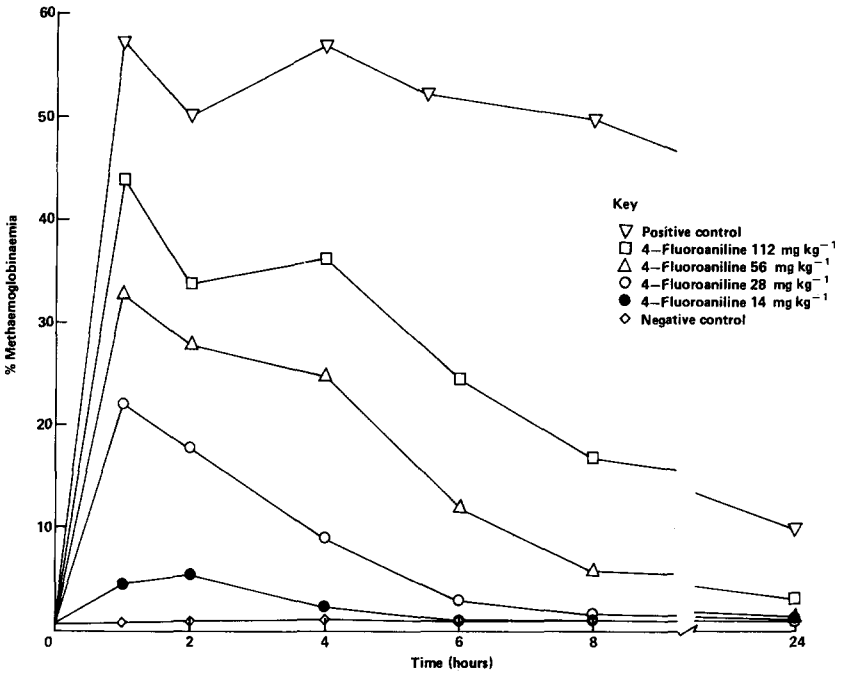

Fig. 3. Variation of methaemoglobinaemia with time and dose after single oral doses of 4-fluoroaniline to rats

Table 2. Half-lives of methaemoglobin at various doses of 2,4-difluoroaniline and 4-fluoroaniline to rats

Dose ($mg\,kg^{-1}$)	Half-life (h)	95% confidence limits		Correlation coefficient
		Upper (h)	Lower (h)	
2,4-Difluoroaniline				
16	2.5	4.5	−4.1	−0.9234
32	1.5	1.8	1.2	−0.9991
64	2.8	5.0	−4.9	−0.9135
128	8.5	27.8	6.0	−0.9142
4-Fluoroaniline				
14	2.3	3.9	−6.1	−0.9482
28	1.7	2.3	1.6	−0.9941
56	2.8	4.1	0.4	−0.9611
112	5.6	9.0	3.9	−0.9452

within 1 or 2 h, indicating that both absorption and metabolism to the oxidised derivatives (Kiese and Uehleke 1961; Kampffmeyer and Kiese 1964) is rapid. The methaemoglobin content also drops quickly, and, with the exception of the top doses, had returned to background levels within 24 h. Pilot studies, where two rats were given the top dose and monitored for two days, showed that the levels

in the animals continued to fall over the second 24 h, indicating that, even at the highest doses administered, a persistent methaemoglobinaemia does not occur. Using the data for the first 8 h after dosing, the half-lives for the disappearance of methaemoglobin, assuming an exponential decay, were calculated and are presented in Table 2. Interestingly it can be seen that the rates of disappearance seem to decrease as the dose increases, although the 95% confidence limits are widely spaced, possibly owing to the relatively poor fit of the data to an exponential curve. This could be due, at the high doses, to enterogastric cycling of the compound. This phenomenon has been reported to occur with aniline (Irons et al. 1980).

The dose-response relationship for both compounds is approximately linear, and would enable an estimation of the dose to be made from the amounts of methaemoglobin, provided the time interval between exposure and blood sampling was known. The limit of detection of exposure in rats, using samples taken when the methaemoglobinaemia is greatest, is about 5 mg kg^{-1}.

It has not been possible to measure methaemoglobin levels in plant workers exposed to DFA or PFA. However, it must be noted that, in the few in-vivo studies reported in the literature, man has been shown to be more susceptible

Table 3. Excretion of 0-sulphate metabolites in the urine of rats dosed orally with 4-fluoroaniline and 2,4-difluoroaniline

Fluoroaniline dose ($mg\,kg^{-1}$)	Collection period after dosing	Urinary volume[a] (ml)	Urinary 0-sulphate excreted (expressed as parent aniline)			
			GC-NTD ($\mu g\,ml^{-1}$)	GC-SIM ($\mu g\,ml^{-1}$)	mg[b]	% of dose given
4-Fluoroaniline						
14	0–24 h	28	178	152	4.6	33
	24–48 h	24.5	6	n/a	0.1	1
28	0–24 h	36.5	406	330	13.4	48
	24–48 h	28.5	14	n/a	0.4	1
56	0–24 h	33	627	708	22.0	38
	24–48 h	21	55	n/a	1.2	2
112	0–24 h	71	543	528	38.0	35
	24–48 h	51	52	n/a	2.7	2
2,4-Difluoroaniline						
16	0–24 h	25	135	104	3.0	19
	24–48 h	20	4	n/a	0.1	1
32	0–24 h	58.5	65	50	3.4	11
	24–48 h	38	7	n/a	0.3	1
64	0–24 h	52.5	140	124	6.9	11
	24–48 h	23.5	27	n/a	0.6	1
128	0–24 h	78.5	182	157	13.3	10
	24–48 h	54	24	n/a	1.3	1

[a] Total from 4 rats
[b] Results from GC-NTD and GC-SIM have been averaged
n/a, Not analysed

than rat to methaemoglobinaemia produced by aniline (Jenkins et al. 1972) and less susceptible to dosing with acetanilide (Lester 1943).

Urinary metabolites

Urinary metabolites were isolated, as the cetylpyridinium salts, from rats dosed with 4-fluoroaniline and 2,4-difluoroaniline. Analysis of the purified salts by nmr and elemental analysis confirmed that these metabolites are the 0-sulphates of 2-amino-5-aminophenol and 2-amino-3,5-difluorophenol respectively (IIi and IIii in Fig. 1).

The results of analysis of urine samples from rats dosed with various amounts of 4-fluoroaniline and 2,4-difluoroaniline are shown in Table 3. The quantities of 0-sulphate metabolites, expressed as parent fluoroaniline equivalents, have been plotted against the dose of fluoroaniline administered (Fig. 4). The excretion of urinary conjugated aminophenols in the male rat after oral administration of 4-fluoroaniline and 2,4-difluoroaniline is rapid. Only very low concentrations of these urinary metabolites were detected on the second day after dosing. These metabolites were not detected in urine from rats given a control, corn oil; or in

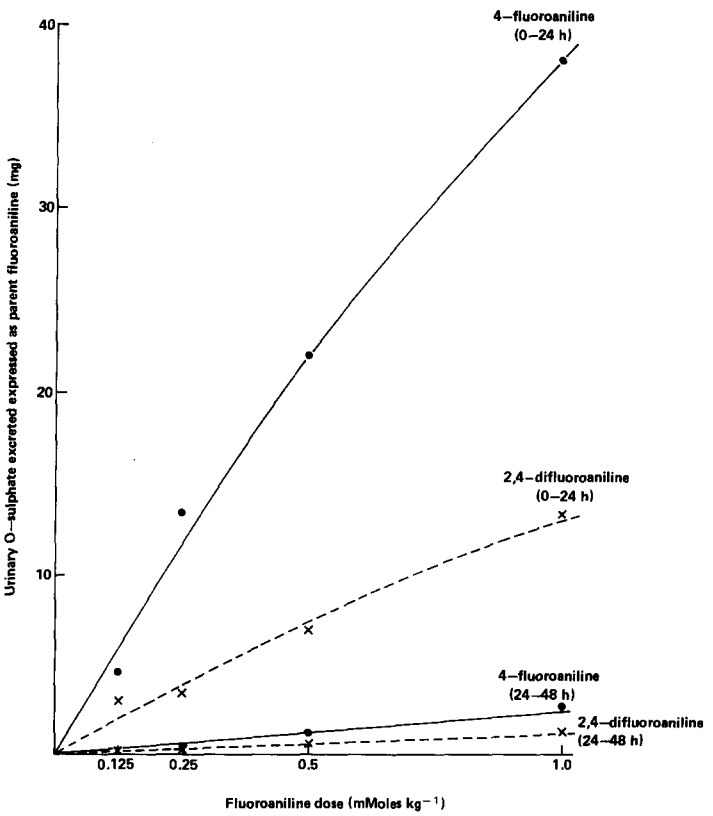

Fig. 4. Relationship between the dose of fluoroaniline administered and the subsequent excretion in the rat of the urinary 0-sulphate metabolite

control human urine samples from unexposed personnel. The limit of detection of the analytical method was of the order of 0.1 μg ml^{-1}, expressed as parent fluoroaniline.

The amounts of these 0-sulphate metabolites excreted in the urine are clearly dose-related, although a smaller proportion of the metabolites (39% and 13% respectively) was excreted from animals dosed with 4-fluoroaniline and 2,4-difluororaniline, compared with a similar study with 3-chloro-4-fluoroaniline (Baldwin and Hutson 1980), where the corresponding 0-sulphate was excreted as 52% of the original dose. Since only 13% of the dose was accounted for in the metabolism of 2,4-difluoroaniline the compound may be excreted more efficiently in another form, and hence a more sensitive method of analysis may be possible for this compound.

Comparison of methaemoglobin and metabolite analysis as monitoring methods

As possible monitoring techniques, the two methods have the following attributes. Metabolite analysis is more sensitive, the limit of detection being about 0.1 μg ml^{-1} of urine, which, if based on a typical daily output of urine, represents a dose of about 0.2 mg to man, assuming man and rat excrete similar proportions of the doses as the 0-sulphate metabolites. The limit of detection using methaemoglobin measurements is about 5 mg kg^{-1} of body weight (again assuming identical responses in man and rat); this, in the average man, is equivalent to a dose of about 350 mg. Metabolite analysis is specific and permits estimation of exposure to more than one aniline at the same time. Methaemoglobin analysis is critically dependent on the timing of sampling and of analysis. The methaemoglobin analysis method has the advantage of not requiring expensive equipment and of ease and rapidity of analysis.

Both techniques suffer from the problem of extrapolation to man. While there are probably differences between rat and man in both metabolic profile and capacity, measurable concentrations of the 0-sulphate metabolites of 4-fluoroaniline and 2,4-difluoroaniline have been observed in urine samples from plant workers engaged in the manufacture of these anilines. These results show that exposure to these anilines has occurred, but do not show how much exposure there was. The relationship between the intake of either aniline and the excretion of the respective 0-sulphate metabolites in the human is at present unknown.

Conclusions

2,4-Difluoroaniline and 4-fluoroaniline are both potent inducers of methaemoglobinaemia in the rat. The amount of methaemoglobin induced is proportional to the dose administered and varies rapidly with the time since dosing. Both compounds are metabolised by rats to the corresponding *ortho*-sulphates, which are excreted in the urine (13% of the dose of 2,4-difluoroaniline, 39% of the dose of 4-fluoroaniline).

Either determination of methaemoglobin content or quantitation of the urinary metabolites could be used as a method for exposure monitoring.

On the basis of the data available, analysis of urine for the 0-sulphate metabolites of 2,4-difluoroaniline and 4-fluoroaniline is the more sensitive method and appears to provide the preferred approach for routine monitoring for exposure of workers to the two compounds. Monitoring methaemoglobinaemia could be useful in the event of an acute exposure, particularly in assessing recovery to normal.

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