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The urine mutagenicity assay system

Studies related to recovery, storage and concentration procedures

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ONG T, STOCKHAUSEN A, ADAMO D, WHONG W-Z. The urine mutagenicity assay system: Studies related to recovery, storage and concentration procedures. *Scand J Work Environ Health* 11 (1985) 45–50. Studies were conducted to determine (i) the effect of storage on the mutagenic activity of urine spiked with known mutagens, (ii) the efficiency of XAD-2 column on the recovery of complex mixtures from spiked urine samples, and (iii) whether the addition of XAD-7 resin to the column and/or the addition of methylene chloride to the elution process increases the recovery of mutagens from urine samples spiked with complex mixtures. Chemically spiked or nonspiked urine samples were concentrated with XAD resin and were tested for mutagenic activity with the Ames *Salmonella*/microsome assay system. The results indicate that the mutagenic activity of chemically spiked urine remains essentially unchanged after 7 d, but it decreases by approximately 50 % for some compounds after 28 d of storage at –70°C. The recovery of mutagens from mutagen-spiked urine varied from 0 % to more than 100 %. Mutagenic activities of concentrates from urine samples spiked with complex mixtures were higher if resin columns were eluted with both methylene chloride and acetone than with acetone alone. A slight increase in mutagenic activity was also found if a mixture of XAD-2 and XAD-7 instead of XAD-2 alone was used to concentrate urine spiked with air particle extracts.

Key terms: complex mixture, mutagen, *Salmonella*, XAD resin.

Workers are often exposed to man-made or naturally occurring chemicals and complex mixtures that may enter the body by means of skin absorption, ingestion, or inhalation. The chemicals can then be activated or deactivated by different enzyme systems. Active forms may bind to deoxyribonucleic acid (DNA) or other macromolecules in the target organs. However, both active and inactive forms may be excreted in the urine. Several animal studies have shown that, when animals are treated with mutagenic carcinogens or chemotherapeutic agents, mutagenic activity can be detected in their urine (3, 5, 11, 17). Increases in mutagenic activity have also been found in urine samples from smokers and from patients treated with chemotherapeutic agents (11, 13, 18, 19, 20, 22, 24). Therefore, urine analysis for mutagenic activity has been applied in occupational settings as an indication of the exposure of workers to mutagenic and potentially carcinogenic compounds (4, 6, 7, 9, 10, 12, 14).

Since the exposure of human subjects to mutagenic compounds is usually at low concentrations, extraction of chemicals from urine is often needed to detect the mutagenic activity. The extraction procedure established by Yamasaki & Ames (24) has been widely adopted by other investigators for urine mutagenesis analysis. In their procedure, urine is concentrated

with an XAD-2 column, and the column is eluted with acetone. The eluted materials, redissolved in dimethyl sulfoxide (DMSO), are tested for mutagenic activity with the Ames *Salmonella*/microsome assay system. XAD-2 was found by Yamasaki & Ames (24) to be better than other resins for the recovery of different mutagens from mutagen-spiked urine samples.

Studies have been conducted in our laboratory to determine (i) the effect of storage on the mutagenic activity of urine (from a nonsmoker) spiked with known mutagens, (ii) the efficiency of XAD-2 column chromatography on the recovery of several complex mixtures and known mutagens from spiked urine samples, and (iii) whether the addition of XAD-7 resin to the XAD-2 column and/or the addition of methylene chloride to the elution process increases the recovery of the mutagenic activity of urine samples spiked with complex mixtures. In the present paper, the results of these studies are presented.

Materials and methods

Chemicals and environmental samples

Benzo(a)pyrene (BaP), dimethylnitrosamine (DMN), and 20-methylcholanthrene (20-MC) were purchased from the Sigma Chemical Co, St Louis, Missouri. 2-Aminoanthracene (2AA) and 2,4,7-trinitro-9-fluorenone (TNF) were purchased from the Aldrich Chemical Co, Milwaukee, Wisconsin, and aflatoxin B₁ (Afl B₁) was obtained from Calbiochem, San

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Diego, California. Airborne particles were collected on glass fiber filters with a Hi-Vol sampler (General Metal Works, EPA Model) on the roof of a local building 10 m above the ground. Diesel emission particles were obtained from the exhaust pipe of a diesel truck, and coal dust was taken from a West Virginian bituminous coal mine.

Sample preparation

Urine samples. Urine samples collected from non-smokers were pooled, filtered, and the pH was adjusted to 6.5. Each pooled sample was divided into several 300-ml subsamples. Except for the control, subsamples were spiked with 2AA (400 µg), BaP (1.2 mg), Afl B₁ (12 µg), DMN (1.2 g), 20-MC (12 mg), TNF (3.6 µg), nitrosated coal dust extract (750 mg), diesel emission particle extract (45 mg), or airborne particle extract (326 mg). All the chemicals or complex mixtures in DMSO were freshly prepared for each experiment before being added to urine samples. Chemically spiked and nonspiked subsamples were concentrated immediately or after storage at -70°C for up to 28 d.

Airborne particle and diesel emission particle extracts. Samples of airborne particles and diesel emission particles were each extracted with methylene chloride for 16 h with vigorous shaking. The extracts were filtered and concentrated with a rotary evaporator to dryness. The dried extracts were redissolved in DMSO.

Nitrosated coal dust extracts. A sample of bituminous coal dust was extracted with methylene chloride for 16 h at room temperature with shaking. The extracted materials were collected by filtration. The residue was extracted a second time with ethyl acetate plus methanol (1:1 volume/volume) under the same conditions. Both extracts were combined and concentrated with a rotary evaporator to dryness. The dry extract was redissolved in DMSO and was nitrosated with sodium nitrite (15 mg/ml) at pH ~ 3.5 for 3 h with shaking (23).

Concentration of urine samples

The procedure of Yamasaki & Ames (24), with modifications, was used for the concentration of urine. Each subsample was passed through a 10-ml XAD-2 or XAD-2 plus XAD-7 (5 ml each) column at a flow rate of 2–3 ml/min. After rinsing with 10 ml of distilled water, the column was flushed with nitrogen to remove the excess water and then eluted with 30 ml of acetone or with 15 ml of methylene chloride followed by 15 ml of acetone. The eluate was concentrated to 0.5 ml at 45°C under a stream of nitrogen. Then 1.2 ml of DMSO was added to each concentrate, which was further evaporated to 1.2 ml. The concentrates were used for mutagenicity studies.

Mutagenesis assays

Chemicals, complex mixtures, and urine concentrates were tested for mutagenic activity with the Ames *Salmonella*/microsome assay system. The plate incorporation and/or preincubation tests with or without S9 activation were employed using tester strain TA98 or TA1535. TA98 was used because the compounds (with the exception of DMN) and mixtures studied are known to be mutagenic to this strain. TA1535 was used only for DMN. The procedures of the plate incorporation test followed those described by Ames and his co-workers (2). In brief, 0.1 ml of tester cells from an overnight culture and 0.1 ml of test material or DMSO (control) were added to 2 ml of molten soft agar containing biotin and a trace amount of histidine. The mixture was overlaid onto a Vogel-Bonner (21) salt minimum agar plate. For the metabolic activation, 0.5 ml of S9 mix (0.05 ml S9) was also added to the soft agar. The S9 was prepared from the liver of Aroclor-1254 (500 mg/kg body weight) pretreated male Wistar rats. In the preincubation test, 0.1 ml of tester cells, 0.1 ml of test material, and 0.5 ml of S9 mix or phosphate buffer were coincubated at 37°C. After 30 min of incubation, 2 ml of molten soft agar containing biotin and a trace amount of histidine were added; this mixture was overlaid onto a Vogel-Bonner salt minimal agar plate. All the overlaid plates were scored for revertants after 2 d of incubation at 37°C.

Results

The effect of storage on the mutagenic activity of nonsmokers' urine spiked with mutagens depended upon the chemical spiked (table 1). The mutagenic activity did not decrease when urine samples spiked with BaP, Afl B₁, or 20-MC were stored for up to 28 d. However, a 50 % decrease in the mutagenic activity was found when urine samples spiked with TNF were stored at -70°C for 28 d.

Based on the mutagenic activity, the recovery of mutagens from nonsmokers' urine spiked with chemicals varied from 0 to over 100 % (table 1). Determination of the recovery percentage was based on the dose-response curves. It should be noted that the dose-response curves were not linear; therefore, the percentages shown are only approximate recoveries. No mutagenic activity could be detected when the urine sample was spiked with DMN, concentrated with the XAD-2 column, and eluted with methylene chloride and acetone. The recovery was around 80 % for 2AA and TNF, but was less than 10 % for BaP and 20-MC. However, at the same concentration, the concentrate from Afl B₁-spiked urine gave four times more revertant colonies than the Afl B₁ stock solution. A follow-up study with Afl B₁ indicated that the increase in mutagenic activity was found only when Afl B₁ was spiked into urine. Urine ex-

Table 1. Effect of storage on the mutagenicity of chemically spiked urine in *S typhimurium* TA98.^a

Chemical	Concentration of spiked chemical (µg/plate)	Revertants per plate		Recovery percentage ^b
		0 d of storage	28 d of storage	
2-Aminoanthracene	10.0	2 212	1 091	80.1
Benzo(a)pyrene	30.0	441	614	9.0
2,4,7-Trinitro-9-fluorenone ^c	0.3	1 050	596	83.4
Aflatoxin B ₁	0.3	1 662	1 329	417.0
20-Methylcholanthrene ^d	0.3	324	496	5.4
Dimethylnitrosamine ^{d,e}	30.0	22	.	0.0

^a Tested by the plate incorporation test. Results are the average of two experiments. The background spontaneous revertants ranged from 24 to 54 per plate.

^b Recovery percentage is based on the ratio of nonspiked (stock solution) and spiked chemical concentrations which gave similar numbers of revertants per plate. The calculation was only for the unstored samples.

^c Tested without S9 activation.

^d The concentration is in milligrams.

^e Assayed by a preincubation test with TA1535.

tract did not affect the mutagenic activity of Afl B₁ (table 2).

The recovery of three complex mixtures from spiked urine was between 20 to 40 % (table 3). In the experiment with different coal dust samples, however, the recovery was only 10 %, a figure which remained relatively constant regardless of the amount of nitrosated coal dust extract spiked into the same amount of urine (table 4). XAD-2 was used for the concentration, and both methylene chloride and acetone were used for the elution of mutagenic compounds for all the studies reported in tables 1–4.

The results of comparative studies between XAD-2 and XAD-2 plus XAD-7 for the recovery of mutagens from urine samples spiked with complex mixtures indicated that a combination of XAD-2 and XAD-7 gave a slight increase in, had no effect on, and gave a slight decrease in the recovery of mutagens from urine spiked with air particle, coal dust, and diesel emission particle extracts, respectively

Table 2. Effect of urine on aflatoxin mutagenesis.^a

Sample tested	Concentration (µg)	Revertants/plate
Aflatoxin B ₁	0.03	218
	0.1	616
	0.3	1 354
	1.0	1 740
Extract of urine spiked with aflatoxin B ₁	0.03	402
	0.1	1 221
	0.3	1 781
	1.0	914
Extract of urine spiked with solvent	0.03	35
	0.1	32
	0.3	35
	1.0	52
Urine extract plus aflatoxin B ₁	0.03	161
	0.1	638
	0.3	1 404
	1.0	1 622

^a TA98 was tested with S9 by the plate incorporation test. The results are averages of two experiments.

Table 3. Mutagenic activity of urine, spiked with a complex mixture, in *S typhimurium* TA98.^a

Complex mixture	Concentration (mg)		Revertants per plate		Recovery percentage ^b
	Stock solution	Spiked urine extract	Stock solution	Spiked urine extract	
Control (dimethyl sulfoxide)	.	.	.	18	.
Air particle extract	2.0	2.7	259	76	30.4
	4.1	5.4	429	159	..
	8.2	10.9	684	247	..
	16.3	21.8	1 047	802	..
Nitrosated coal dust extract	2.3	3.1	65	46	40.5
	4.7	6.2	183	89	..
	9.4	12.5	398	132	..
	18.8	25.0	1 138	364	..
Diesel emission particle extract	0.03	0.1	357	150	19.0
	0.05	0.2	688	321	..
	0.15	0.4	1 345	622	..
	0.50	0.8	1 744	1 035	..

^a Results are averages of two experiments by the plate incorporation test. No S9 was used.

^b The recovery percentage shown is an average of four different tested concentrations.

Table 4. Comparison of the mutagenic activity of concentrates from urine samples spiked with different amounts of nitrosated coal dust extract (NCDE).^a

Sample tested	Concentration used (mg)	Revertants/plate	Recovery percentage
Nitrosated coal dust extract	1.03	28	.
	3.13	62	.
	10.31	138	.
	31.25	336	.
Urine concentrate 1 (0.25 ml NCDE spiked)	1.47	18	..
	4.46	30	..
	14.7	43	12.9
	44.6	63	7.2
Urine concentrate 2 (0.50 ml NCDE spiked)	2.95	29	..
	8.93	28	..
	29.5	60	10.2
	89.3	99	7.6
Urine concentrate 3 (1 ml NCDE spiked)	5.89	23	..
	17.86	37	..
	58.9	84	8.2
	178.6	161	6.8
Solvent control	.	19	.
Urine extract control	.	20	.

^a TA98 was tested without S9 by the plate incorporation test. The results are based on one experiment in duplicate.

(table 5). The addition of methylene chloride to the elution process appears to increase the recovery of mutagens from urine spiked with all three complex mixtures. In the case of diesel emission particles, an approximately fourfold increase was found when both acetone and methylene chloride were used for the elution instead of acetone alone (table 6).

Discussion

The reported studies indicate that mutagens such as BaP, Afl B₁, and 20-MC are stable in urine. The mutagenicity of TNF appeared to be stable in urine for 7 d at -70°C (data not shown); however, the mutagenic activity was only 50 % after 28 d of storage. Putzrath et al (15) found that the mutagenic activity of extracts from smokers' urine remained stable for three months at -20°C. Therefore, the stability of mutagens in urine seems to vary from compound to compound.

Yamasaki & Ames (24) have studied the recovery of various mutagens from mutagen-spiked urine samples by XAD-2 columns. The studies reported in the present paper were extended to complex mixtures and other known compounds. Complex mixtures were used in the study because workers are often exposed to complex mixtures rather than to a single compound in occupational settings. The percentage of mutagen recovery, based on the mutagenicity data, appears to vary from experiment to experiment or from sample to sample. For instance, the recovery of nitrosated coal dust extract in one sample was 40 %

Table 5. Comparison between XAD-2 and XAD-2 plus XAD-7 for the recovery of mutagens from urine samples spiked with complex mixtures.^a

Complex mixture	Concentration used (mg)	Revertants/plate
Air particle extract		
XAD-2 + XAD-7	0.77	40
	2.33	85
	7.77	226
	23.32	370
XAD-2	0.77	38
	2.33	68
	7.77	177
	23.32	345
Nitrosated coal dust extract		
XAD-2 + XAD-7	2.38	33
	7.14	50
	23.80	123
	71.40	205
XAD-2	2.38	30
	7.14	49
	23.80	114
	71.40	204
Diesel particle extract		
XAD-2 + XAD-7	4.6	267
	14.0	617
	46.0	1 292
	140.0	1 903
XAD-2	4.6	642
	14.0	1 268
	46.0	1 968
	140.0	2 434
Solvent control	.	19
Urine control		
XAD-2 + XAD-7	.	21
XAD-2	.	43

^a TA98 was tested without S9 by the plate incorporation test. The results are averages of two experiments.

(table 3); however, with another sample it was less than 10 % (table 4). The fluctuation of the recovery with the same compound or complex mixture may be expected because so many factors are involved, ie, concentration with resin, elution with solvent, evaporation of solvent, and mutagenesis testing. This is likely to be one of the reasons why the recovery percentage of a given compound differed between the studies. The low recovery of complex mixtures and certain compounds as indicated in tables 1 and 3 is probably not due to overloading of the column with the spiked material but is likely due to the low efficiency of XAD-2 for certain chemical agents. The concentration procedure used did not appear to recover DMN. Failure to recover DMN may be due to its high solubility in the aqueous solvent or its high volatility.

The results with Afl B₁ indicate that urine of nonsmokers can enhance the mutagenic activity of this compound. It has been shown that the extract of urine from smokers, but not nonsmokers, has a syn-

Table 6. Comparison between acetone and acetone plus methylene chloride for the recovery of mutagens from urine samples spiked with complex mixtures.^a

Complex mixtures	Concentration used (mg)	Revertants/plate
Air particle extract		
Methylene chloride followed with acetone	0.77	51
	2.33	86
	7.77	179
	23.32	363
Acetone	0.77	36
	2.33	49
	7.77	114
	23.32	282
Nitrosated coal dust extract		
Methylene chloride followed with acetone	2.38	57
	7.14	94
	23.80	218
	71.40	390
Acetone	2.38	46
	7.14	70
	23.80	162
	71.40	260
Diesel particle extract		
Methylene chloride followed with acetone	4.6	149
	14.0	553
	46.0	1 468
	140.0	2 258
Acetone	4.6	70
	14.0	111
	46.0	320
	140.0	672
Solvent control	-	24
Urine control		
Acetone	-	36
Methylene chloride followed with acetone	-	37

^a TA98 was tested without S9 by the plate incorporation test. The results are averages of two experiments. Urine samples were from a nonsmoker.

ergistic effect on the mutagenic activity of 2AA (8). Urine extract from nonsmokers was found by Aeschbacher & Ruch (1) to inhibit the mutagenic activity of 2-aminofluorene and daunomycin. It is interesting to note that the enhancement of Afl B₁ mutagenesis is not due to XAD-2 extractable material, but most likely it is caused by aqueous soluble substances.

XAD-2 is a nonpolar resin, while XAD-7 is moderately polar. A combination of XAD-2 and XAD-7, in an attempt to collect more chemical species, was used by Rappaport et al (16) to extract mutagens from waste water. The comparison study of XAD-2 and XAD-2 plus XAD-7 in this report indicated that the combination of XAD-2 and XAD-7 increased and decreased the recovery of mutagens from airborne particle extract and diesel emission particle extract, respectively. The increase may not be significant however. No doubt XAD-2 alone would be a better resin for exclusively or primarily nonpolar organic materials. However, a combination of XAD-2 and

XAD-7 may be a better system for some chemicals or complex mixtures.

In their mutagenicity study of urine from chemical workers, Kriebel and his co-workers (10) used both acetone and methylene chloride to elute XAD-2 columns. They showed that the mutagenic activity frequently appeared in either or both solvent extracts. Based on the three complex mixtures studied in the present investigation, elution of resin columns with methylene chloride and acetone gave higher mutagenic responses than with acetone alone. The difference was highly significant with the diesel particle extract. Since methylene chloride is relatively nonpolar while acetone is polar, elution of the column with both methylene chloride and acetone may be able to extract mutagens with different polarities and, therefore, give a higher mutagenic response. It has to be pointed out, however, that methylene chloride was the solvent used for the extraction of organic materials from complex mixtures. Hence, an increase in the mutagenic response may be expected if methylene chloride is used in the elution process. It is interesting to note that most mutagens extractable from airborne particles and coal dust, but not from diesel emission particles, with methylene chloride are also extractable with acetone.

The results from this study suggest that (i) urine samples can be stored for at least one week before processing and testing for mutagenic activity, (ii) the addition of methylene chloride to the elution process increases the recovery of mutagenic material, (iii) the addition of XAD-7 may increase the recovery of mutagenic materials for certain environmental samples, and (iv) the recovery percentage (based on mutagenesis testings) varies highly (0 to over 100 %) from chemical to chemical.

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