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SORPTION OF ORGANIC COMPOUNDS FROM URINE IN MUTAGENICITY TESTING: CHOICE OF SORBENT

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SUMMARY

The choice of sorbents for urine mutagenicity testing was investigated. ^{14}C-Labelled compounds were dissolved in urine and extracted from it by sorption with seven different sorbents singly or in combination. The compounds were desorbed with acetone. Sorption was examined under various experimental conditions. Except for two strong ionic compounds satisfactory extraction was achieved with a combination of three sorbents: Amberlite XAD-2, XAD-7, and Sep-Pak Silica. Filtration of the urine or contact of it with the walls of the experimental equipment could lead to substantial losses. Implications of these results for urine mutagenicity testing and possibilities for further optimization of the method are discussed.

INTRODUCTION

Several recent studies have shown that the urine of people exposed to certain work environments, or to cytostatics or tobacco tar (smokers) [1—5] may contain mutagenic substances. In most of this work it was necessary to extract and concentrate the mutagens before they could be studied. For this purpose two techniques were applied: liquid—liquid extraction with dichloromethane or other non-polar solvents; and sorption of the mutagens on Amberlite XAD-2 resin. Although sorption is the more frequently used technique, it has so far not been fully evaluated. One aspect that deserves further study is the type of sorbent used. Yamasaki and Ames [6] were the first to use Amberlite XAD-2 in their experiments with urine of cigarette smokers, and their results led others to follow suit. However, since mutagens are not necessarily chemically similar, the question remains whether other sorbents or combinations of sorbents may not give better results. To answer this question was the first aim of this study, justified by the possible use of urine in routine monitoring of people exposed to mutagenic compounds in their environment.

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We studied the sorption and desorption of ^{14}C-labelled compounds from urine, using seven different sorbents, singly or in combinations, under a variety of experimental conditions. Another aspect studied was the adsorption of the model compounds on the filters and walls of the experimental equipment.

METHODS AND MATERIALS

Sorbents

The sorbents and their salient properties are listed in Table I. A more detailed description is given by Dressler [7] and Saner et al. [8].

The Amberlite XAD sorbents (supplied by Rohm and Haas) were purified by Soxhlet extraction with methanol (24 h) and diethyl ether (24 h) and stored under methanol. They were packed in stainless-steel columns (180 mm, 3 ml; Chrompack, The Netherlands) and later in polyvinylidene fluoride columns, made in cooperation with the TNO Institute for Plastic and Rubber Research (95 mm, 3 ml).

The Sep-Pak sorbents were supplied (Waters Assoc.) already packed in polyethylene cartridges (Sep-Pak Silica 20 mm, 1.5 ml; Sep-Pak C_{18} 10 mm, 0.8 ml).

Just before use all columns were eluted with 50 ml of acetone and 50 ml of distilled water at a flow-rate of 4 ml min^{-1}.

TABLE I

SORBENTS USED

Sorbent	Chemical name*	Surface area per unit weight* ($m^2\ g^{-1}$)	Mean pore diameter* (μm)
Sep-Pak C_{18}	Octadecylsilane	400	—
Sep-Pak Silica	**	**	**
XAD-1	Styrene divinylbenzene copolymer	100	0.01
XAD-2	Styrene divinylbenzene copolymer	300	0.009
XAD-4	Styrene divinylbenzene copolymer	725	0.004
XAD-7	Methacrylate polymer	450	0.009
XAD-8	Methylmethacrylate polymer	160	0.0225

*Information of the manufacturer.
**No information available.

^{14}C-Labelled compounds

The following ^{14}C-labelled compounds were used: benzene, phenol, benzo(*a*)pyrene, Aroclor 1254, ethylenediamine, dioctylphthalate, chloroacetic acid, *p*-chlorophenol, *p*-nitrophenol, 3,4-dichloroaniline, didecyldimethylammonium bromide.

Except for didecyldimethylammonium bromide, which was synthesized at our institute, all compounds were supplied by the Radiochemical Centre (Amersham, U.K.). Stock solutions of the compounds were made in acetone or distilled water. Of each solution 15 μl were mixed with 50 ml of urine.

Urine samples

Urine was collected in batches from several male employees of our institute who neither smoked nor took medicine. Each batch was at once chilled to 0—4°C upon collection, and its pH was measured: the mean pH was 5.8 ± 0.5. Just before addition of the labelled compounds, the urine was filtered over a regenerated cellulose membrane filter (Sartorius SM 11604, 0.8 μm).

Solvents

All organic solvents (glass-distilled or HPLC grade) were supplied by Rathburn Chemicals (U.K.).

Sorption experiments

Unless noted otherwise in the text, the set-up of the sorption experiments was the same as that used by Yamasaky and Ames [6]. A 50-ml volume of urine was sucked into an evacuated glass vial and was pumped through the column by increasing the air pressure in the vial. The flow-rate was regulated by changing the air pressure (mean value 4 ml min^{-1}). This method of filling the sample vial reduces the amount of gas dissolved in the urine, and promotes better contact between urine and the sorbent.

The bed volume was 3 ml for the XAD sorbents, 0.8 ml for Sep-Pak C_{18} and 1.5 ml for Sep-Pak Silica. Aliquots of urine (2 ml) were taken before and after passage through the column to determine the ^{14}C-labelled compounds by liquid scintillation counting. The sorption percentage is the difference between these amounts as a percentage of the amount before passage.

Desorption experiments

The sorbed compounds were desorbed immediately after passage of the urine, by passing through the column in the opposite direction two 5-ml portions of acetone at a flow-rate of 3 ml min^{-1}. After passage, the two acetone portions were kept separate, and their content of ^{14}C-labelled compound was measured. The desorption conditions were chosen based on the results obtained with ^{14}C-labelled compounds in distilled water which showed that backflush desorption is to be preferred to straight desorption. The desorption percentage is the amount of ^{14}C desorbed as a percentage of the amount sorbed.

RESULTS

Combining sorbents for best results

In a series of experiments we studied the sorption of four compounds (benzo(*a*)pyrene, benzene, phenol and Aroclor 1254) by all sorbents at three urine pH values (pH 2, pH 10 and the physiological pH of urine). The results of these experiments allowed us to select sorbents for further experiments. In these we chose conditions believed to be less favourable to sorption: the concentration of the compound, the volume of the urine, and the flow-rate of the urine were all increased; the volume of the sorbent (bed volume) was decreased. In two experiments the removal of radioactivity from the sorbent was measured when another portion of urine, without labelled compound, was

pumped through the column. A summary of the results is given in Table II.

Phenol is best sorbed by XAD-7 and XAD-4 at physiological pH. At pH 2 the sorption was negligible; at pH 10 it was a little less than at the physiological pH of urine.

The sorption of benzene is not very pH-dependent, and none of the XAD resins emerges as being best for the sorption of this compound. Benzo(*a*)pyrene is best sorbed from urine by Sep-Pak C_{18} and Sep-Pak Silica. The sorption is

TABLE II

PERCENTAGE SORPTION FROM URINE OF FOUR ^{14}C-LABELLED COMPOUNDS WITH VARIOUS SORBENTS UNDER FIVE (I—V) EXPERIMENTAL CONDITIONS

Experiments: (I) basic sorption set-up with lower concentration, (II—V) higher concentration, (II) experiment with distilled water, (III) volume of urine increased to 300 ml, (IV) flow-rate increased to 40 ml min^{-1}, (V) bed volume decreased to one-third with XAD or one-half with Sep-Pak. The XAD resins were packed in stainless-steel columns.

	I	II*	III*	IV	V
Phenol (pH not adjusted, 2 and 200 $\mu g\ l^{-1}$)					
Sep-Pak Silica	5				
Sep-Pak C_{18}	4				
XAD-1	71	61			
XAD-2	69	81			
XAD-4	92	96	66	42	72
XAD-7	94	93	65	81	60
XAD-8	95	86	49		
Benzene (pH 2, 0.6 and 60 $\mu g\ l^{-1}$)					
Sep-Pak Silica	31				
Sep-Pak C_{18}	88	80			
XAD-1	98	99	76		
XAD-2	95	99	77	94	71
XAD-4	93	99	65	97	63
XAD-7	99	99			
XAD-8	98	92			
Benzo(*a*)pyrene (pH 2, 9 and 18 $\mu g\ l^{-1}$)					
Sep-Pak Silica	96	100	66	99	
Sep-Pak C_{18}	93	99	96	96	98
XAD-1	71	46			
XAD-2	61	39			
XAD-4		61	37		
XAD-7		77	56		
XAD-8		88	74		
Aroclor 1245 (pH 2, 2 and 50 $\mu g\ l^{-1}$)					
Sep-Pak Silica	98	99	94	76	
Sep-Pak C_{18}	83	98	87	71	71
XAD-1	71	79			
XAD-2	56	76			
XAD-4	63	74			
XAD-7	83	81			
XAD-8	78	79			

*Experiment in which an extra portion of urine or water was pumped through the sorbent.

TABLE III

PERCENTAGE SORPTION FROM URINE OF ^{14}C-LABELLED COMPOUNDS WITH VARIOUS SORBENTS

The basic sorption set-up was used. The XAD resins were packed in polyvinylidene columns. The pH of the urine was not adjusted.

	Sep-Pak Silica	Sep-Pak C_{18}	XAD-1	XAD-2	XAD-4	XAD-7	XAD-8
Ethylene diamine ($18\ \mu g\ l^{-1}$)	14	5	16	18	21	17	13
Dioctylphthalate ($250\ \mu g\ l^{-1}$)	90	83*	10	11	9	13	15
Chloroacetic acid ($8\ \mu g\ l^{-1}$)	4	1	2	4	5	4	7
p-Chlorophenol ($39\ \mu g\ l^{-1}$)	10	11	84	85	87	89	88
p-Nitrophenol ($12\ \mu g\ l^{-1}$)	6	3	77	89	98	100	100
3,4-Dichloroaniline ($20\ \mu g\ l^{-1}$)	16	90**	74	74	70	67	70
Didecyldimethylammonium bromide ($20\ \mu g\ l^{-1}$)	93	77	85	85	85	86	87

*Duplicate value was 26 (see also Table V).
**Duplicate value was 46.

somewhat better at pH 2 or pH 10 than at the natural pH. Likewise, Sep-Pak cartridges are the best sorbents for Aroclor 1254. Also with this compound the sorption is not very pH-dependent.

The extra portion of urine without ^{14}C-labelled compound resulted in a considerable loss (> 10%) of phenol when XAD-1, -2 or -8 were used and of benzene when Sep-Pak C_{18} was used.

The sorption of the other labelled compounds was investigated less thoroughly. Using the basic sorption set-up, we extracted them with each of the sorbents only at the physiological pH of urine. The ionic compounds were also extracted at pH 2 or 10. Results are given in Table III. The sorption of ethylene diamine did not improve at a higher pH; that of chloroacetic acid increased to 14—28% with the XAD resins when the pH decreased to 2.

In the next series of experiments all the labelled compounds were sorbed on combinations of two, three or four sorbents; the pH of the urine was not adjusted. The choice of those sorbents was based on the earlier experiments: Sep-Pak Silica, Sep-Pak C_{18}, XAD-2 and XAD-7. In this way it could be established whether two suboptimal sorbents could replace the optimal one, and whether the pH-dependence of the extraction was reduced by combination. Results with combinations of suboptimal sorbents are shown in Table IV.

It is clear from this table that a combination of XAD-2 and XAD-7 is sufficient for almost all compounds. To reach a high sorption percentage with benzo(*a*)pyrene the Sep-Pak Silica can be added to the two XAD sorbents. The

two strong ionogenic and polar compounds are poorly sorbed even with a combination of all four sorbents (10—20%). With this combination the sorption of the other compounds lies in the 90% range. This was also the case when the combination XAD-2—XAD-7—Sep-Pak Silica was used.

TABLE IV

PERCENTAGE SORPTION FROM URINE OF ^{14}C-LABELLED COMPOUNDS WITH COMBINATIONS OF SORBENTS

Apart from the use of more sorbents, the basic sorption set-up was used. The pH of the urine was not adjusted. The XAD resins were packed in polyvinylidenefluoride columns.

Compound	Concentration ($\mu g\ l^{-1}$)	Combination of sorbents*	Percentage sorption
Phenol	200	C_{18}—XAD-2	38
		C_{18}—XAD-2—Sil	45
Benzene	60	XAD-2—XAD-7	34
Benzo(*a*)pyrene	18	C_{18}—XAD-2—XAD-7—Sil	79
Aroclor 1254	50	XAD-2—XAD-7	90
Ethylenediamine	18	C_{18}—XAD-2—XAD-7—Sil	28
Dioctylphthalate	250	XAD-2—XAD-7	96
Chloroacetic acid	8	C_{18}—XAD-2—XAD-7—Sil	10
p-Chlorophenol	39	C_{18}—Sil	14
p-Nitrophenol	12	C_{18}—Sil	6
3,4-Dichloroaniline	20	C_{18}—Sil	51
		C_{18}—XAD-7—Sil	85
Didecyldimethylammonium bromide	20	All combinations	>90

*C_{18} = Sep-Pak C_{18}; Sil = Sep-Pak Silica.

TABLE V

PERCENTAGE DESORPTION OF ^{14}C-LABELLED COMPOUNDS FROM VARIOUS SORBENTS

The concentrations of the compounds were equal to those listed in Table IV. The XAD resins were packed in stainless-steel columns.

Compound	Sorbent	pH*	Adsorption	Desorption	Total extracted**
Benzene	XAD-2	2	95	87	83
Phenol	XAD-4	n.a.	82	77	63
Benzo(*a*)pyrene	Sep-Pak C_{18}	2	96	63	60
Aroclor 1254	Sep-Pak Silica	2	91	104	91
Dioctylphthalate	Sep-Pak C_{18}	n.a.	21	96	20
Chloroacetic acid	XAD-7	2	29	68	20
p-Chlorophenol	XAD-4	n.a.	90	77	69
p-Nitrophenol	XAD-4	n.a.	84	79	66
3,4-Dichloroaniline	XAD-2	n.a.	94	75	71
Didecyldimethylammonium bromide	XAD-4	n.a.	74	70	52

*n.a. = natural pH of urine, not adjusted.
**Percentage of the amount of compound present in the urine that is extracted.

Desorption of the sorbed compounds

Table V shows the results of a number of experiments in which we measured desorption of the labelled compounds. The results indicate that, for optimum desorption, 10 ml of acetone are sufficient. Nearly all the radioactivity was desorbed by the first 5 ml of acetone. It is not clear, however, whether the proportion not desorbed remains behind in the sorbent or on the walls of the sampling vial.

Adsorption on glass, cartridge material and membrane filter material

In the adsorption experiments the urine inevitably comes into contact with materials that are not sorbents, but which may nevertheless act as adsorbents. We briefly investigated the resulting spurious sorption in experiments in which such materials were "exposed" to the urine in the same way as in the basic set-up. Table VI lists the results, which show that a considerable proportion of a compound can be lost through spurious adsorption. Adsorption experiments with stainless-steel columns showed that adsorption of benzo(*a*)pyrene or Aroclor 1254 from distilled water was negligible. The adsorption of benzo(*a*)pyrene with the cartridge material is very high, which can account for the high percentages found with the complete Sep-Pak Silica.

TABLE VI

ADSORPTION OF ^{14}C-LABELLED COMPOUNDS ON GLASS, CARTRIDGE MATERIAL AND MEMBRANE FILTERS

The concentrations of the compounds were equal to those listed in Table IV.

Compound	pH*	"Adsorbent"**	Percentage adsorption	Percentage desorption
Benzene	2	Glass	24	
Phenol	n.a.	Glass	1	
Benzo(*a*)pyrene	2	Glass	14	
		Cartridge	93	
		Filter	50	85
Aroclor 1254	2	Glass	22	
		Cartridge	18	
		Filter	33	111
Dioctylphthalate	n.a.	Filter	44	100

*n.a. = pH of urine not adjusted.

**Glass = vial used in the sorption experiment; cartridge = empty Sep-Pak Silica cartridge; filter = regenerated cellulose membrane filter.

DISCUSSION

An important aspect of mutagenicity studies with urine from humans, thought to have been exposed is the uncertainty about the identity of the mutagen that has to be extracted and concentrated. Mutagens occur in widely different classes of chemicals which implies that the techniques applied should extract a broad spectrum of chemicals. Available techniques are liquid—liquid partition and sorption. With the former the polarity of the extracted

compounds depends on the solvent and is thus rather low because partition must be possible; the latter technique has the advantage that sorption can be varied by changing the type of sorbent used. Combination of appropriate sorbents will enable the extraction of organics of widely varying properties.

The results presented in this paper illustrate the suitability of sorption for several classes of compounds. Except for compounds that are both ionic and polar (ethylenediamine and chloroacetic acid), compounds could be extracted satisfactorily by a combination of sorbents followed by desorption with acetone. It follows that, if one wishes to extract a wide range of mutagens from urine, a combination of sorbents is superior to a single sorbent (e.g. XAD-2).

Most compounds were not fully desorbed by acetone; this phenomenon could be explained by spurious adsorption on materials other than the sorbents themselves. Special care must be taken in filtering the urine, which may result in the loss of a large proportion of the material one wishes to isolate.

The results show that satisfactory extraction of mutagens from urine can be achieved with the combination XAD-2—XAD-7—Sep-Pak Silica.

Urine can be regarded as a rather concentrated solution of polar organic compounds. Their presence may limit the extraction of mutagens to those having a lower polarity, because they may cause toxicity problems in mutagenicity tests, they necessitate large bed volumes to avoid overloading of the sorbent, and they may limit the possible concentration factor of the mutagens.

Attempts aimed at the sorption of more polar mutagens could result in too big a share of the "natural" polar urine constituents in the extract, which in turn could reduce the advantage of being able to sorb the more polar mutagens.

Should urine be found to contain mutagens in such low concentrations — compared to those in our experiments — that it is necessary to aim at higher concentration factors, one might increase the urine volume. This may well cause part of a mutagen already sorbed to be supplanted by the larger amount of "natural" urine constituents that now passes the sorbent. Increasing both bed volume and urine volume, however, leads to sorption of larger amounts of "natural" urine constituents, thus limiting the concentration factor.

These considerations make clear that the polarity of the mutagens in urine that can be extracted and the concentration factor that can be reached are both limited by the "natural" polar urine constituents. Further investigations are therefore necessary to establish optimum experimental conditions for extraction in urine mutagenicity studies. The results of this study indicate which combination of sorbents should be used.

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