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ANALYTIC METHODS FOR BIOCHEMICAL INDICATORS OF RADIATION INJURY

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The development of biochemical indicators of radiation damage has been seriously hampered so far by the lack of pertinent information from irradiated man. Moreover, the fortunately rare accidents in nuclear industry have not been fully exploited with respect to analyzing all compounds of interest. One reason for this failure has been that adequate analytic methods were not available in the institutions treating the patient.

This paper attempts to assemble the analyses which in the opinion of the authors, appear most suitable to assess potential biochemical indicators of radiation damage. Several of these methods were developed or modified in this laboratory. It should be understood that the choice of substances tested as well as of the methods utilized represents a compromise between the most sensitive and specific method available and the one which will yield useful results with a reasonable effort. The dilemma may be illustrated for the case of deoxycytidine. This substance—an interesting indicator present in rats—is excreted by man only in minute amounts (<10 μg/day). A specific and sensitive method for deoxycytidine would require at least three times more work than the one suggested here. Although the latter one gives erroneously

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high results in normal urine it allows to recognize a significant increase (2—4times) in deoxycytidine excretion. Nevertheless, the authors are conscious of the fact that their judgement in these matters may be wrong and this paper is intended only as an impulse for criticism and improvement, in order to arrive at a set of recommendations for compounds to be measured and methods to be used at nuclear accidents.

Methods

General comments

Destruction of delicate compounds present in urine must be avoided. Transport of urine, if necessary, should be done in the frozen state, and, before freezing, urine should be distributed among three samples of about 15 ml each in order to avoid refreezing after thawing. Preliminary experiments indicate that short term freezing affects the enzyme activity only to a minor extent. Nevertheless, the enzyme tests should be performed without delay upon receipt of the urine and should be followed by the separation of biogenic amines on Biorex 70 and the extraction of 5 hydroxyindolacetate. Moreover, the determination of the biogenic amines should be completed on the day of separation. Assay of the various compounds separated on the Biorex 50 column will take more than one day because of the delay due to drying and extraction of paper chromatograms. Two or three experienced technicians should be able to accomplish all tests within 3 days; a single technician will require about 5 days.

The equipment needed for the determination is that usually available in a biochemical laboratory. The spectrophotometer (Cecil UV spectrophotometer) should have a semimicrocell requiring a fill of 1 ml or less. A spectrofluorometer is needed for determining dopamine, histamine, spermidine and hippuric acid and is preferable to a filter instrument for the other compounds. The data have been obtained with a Turner 430 using 3 ml round cuvettes. An analysis of all urinary amino acids including β-amino isobutyrate is desirable after a nuclear accident. No method has been included, however, since details depend on the analyzer in use in the respective laboratory.

A list of the potential indicators, together with normal values as extracted from the literature, are presented in the Table. All values are related to excretion of creatinine, and represent μmole/mg creatinine (for ions, sulfate and total amino acids), μg/mg creatinine for the other compounds and mU/mg creatinine for the enzymes. Although such reference to creatinine has its drawbacks, it is the only means feasible to obtain reproducible data in animals.

Table

Normal values in human and rat urine

	Man, reported in literature	Man, as determined by methods proposed	Rat, as determined by methods proposed
Na^+ (μmole/mg creatine)	100	110	90
K^+	50	40	45
Sulfate, total	20	20	40
Sulfate, free	14	15	20
Creatinine (mg/ml)	1.5	1.5	1 (low, due in part to dilution from washing)
Total amino acids (μmole/mg creatinine)	12	15	10
Taurine (μg/mg creatinine)	90	140	300
Creatine	20	10	100
δ-aminolevulinic acid	2.0	2.5	2
Hydroxyproline	15	30	80
Hippuric acid	300	300	350
Spermidine	0.65 (total)	0.12 (free)	0.16 (free)
Uric acid	300	440	250
Pseudouridine	80	100	120
Deoxycytidine	0.003	$\leqslant$0.01	50
Xanthurenic acid	1.0	4	4
Kynurenic acid	2.0	5	7
Indoxylsulfate	50	50	250
N-methyl-nicotinamide	3.5	2.0	3
N-methylpyridone carboxamide	15	30	20
Sialic acid	35	80	200
Noradrenalin	0.08	0.07	0.15
Dopamine	0.40	0.20	0.50
Serotonin	0.10	0.15	0.30
5-hydroxyindol acetic acid	1.5	1	1
Histamine	0.02	0.05	0.05
Amylase		0.70	10
RNA ase		2	4
Proteolytic activity	20	10	20
β-glucuronidase		0.2	2.5

Values for ions, sulfate and amino acids are given in μmole/mg creatinine, for other compounds as μg/mg creatinine and for enzymes as mU/mg creatinine

Many compounds are excreted in greater amounts by rat than by man (Table). This is well known for deoxycytidine and creatine but is also marked for taurine, sialic acid, kynurenic acid, amylase and β glucuronidase. Moreover, our values for certain compounds (deoxycytidine, kynurenic acid, xanthurenic acid) are significantly larger than those reported in the literature. This is probably due to the lower specificity of our methods and could be avoided only at the expense of considerably more work (additional column separation) which would make the techniques impracticable after an accident.

Preliminary separations

Preparation of resins. Prepare Biorex 50 Wx 8 H^+ 200—400 mesh (Dowex 50) by treating the resin successively with several volumes of 2 N NaOH, H_2O, 2 N HCl and H_2O to neutrality. Fill to a height of 7 ml into 10 ml plastic syringes which have a polypropylen filter disk (RCM 1000, Mannesmann) on the bottom and a stopcock at the outlet. Wash column with 30 ml H_2O before use.

Prepare Biorex AG 1×8 formate 200—400 mesh (Dowex 1) by treating it with 2 N NaOH, H_2O, 2 N formic acid, H_2O. Fill into 2 ml plastic syringes to 0.7 ml, wash with 5 ml H_2O, drain.

Prepare Biorex 70 Na^+ 100—200 mesh by washing resin with 2 N HCl, H_2O, 2 N NaOH, H_2O, phosphate buffer pH 7.0 (9.25 g $K H_2PO_4$ 2 H_2O, 4.27 g $Na_2H PO_4$ 2 H_2O, 1 g EDTA/l). Store in H_2O containing 1 g/l EDTA and adjusted to pH 7.0. For biogenic amines fill into 5 ml plastic syringes to a height of 3 ml and wash with 10 ml EDTA H_2O.

Prepare Biorex AG 1 OH^- by treatment with 2 N NaOH and H_2O. Prepare a slurry of Biorex AG 1 OH^- as well as of Biorex 50 H^+ 1/1 with water. For methylpyridone carboxamides fill into 2 ml plastic syringes successively 0.1 ml AG 50, 0.4 ml AG 1, 0.2 ml AG 50, 0.5 ml AG 1 and 0.25 ml AG 50. Let resin settle between additions to obtain sharp zones, wash column with 20 ml of water, drain.

Separation on Biorex 50 H^+. Spike one 10 ml urine sample with the following standards: 2000 μg taurine, 2000 μg indoxylsulfate, 2000 μg pseudouridine, 100 μg xanthurenic acid, 100 μg kynurenic acid, 30 μg N-methylnicotinamide, 50 μg delta-aminlevulinic acid, 1 μg (in rats 500 μg) deoxycytidine. Prepare the other samples by adding to 10 ml urine 0.1 μCi (of pseudouridine ^{14}C and 1 μCi of deoxycytidine ^{3}H. Apply the 10 ml to Biorex 50 columns, discard the initial 5 ml of effluent, then collect and wash column with 15 ml of water, yielding *Fraction A (20 ml)*. Remove for determinations: 2×20 μl for taurine and 2×500 μl (2×100 μl for rats) for indoxylsulfate. Apply 200 μl to 2.5 cm

wide strips of Whatman 3 MM paper. Develop by descending chromatography with isopropanol /NH_4OH/H_2O[20/1/2] for 48 hours. Localize, the pseudouridine band under ultraviolet light with the help of a reference strip, cut out, concentrate material at one edge by letting water rise 2 times, dry and elute the edge of the paper with 3 ml of H_2O.

Wash column with 100 ml of 0.2 N HCl (16.4 ml HCl/l) followed by 10 ml of H_2O and 10 ml of NH_4OH (1 part conc. NH_4OH, 4 parts H_2O). Discard these effluents. Elute with 10 ml of the NH_4OH solution. This eluate should turn alkaline during the first 2 to 3 ml, otherwise the elution volumes must be adjusted accordingly.

Fraction B (10 ml): Remove 4×200 µl for determination of kynurenic and xanthurenic acid. Dry rest under hood, redissolve in 500 µl of H_2O fraction B 1, remove 3×20 µl for delta aminolevulinic acid, pass remainder through Biorad AG 1 formate column and wash with 2×0.5 ml of water yielding.

Fraction C (1.5 ml): Remove 3×50 µl for N-methylnicotinamide dry rest, dissolve in 300 µl of 50 % methanol, apply to 2.5 cm wide strips of acid washed Whatman 3 MM paper and develop descendingly in ethyl acetate/formic acid/H_2O [15/10/15] overnight, localize under ultraviolet light with the help of a reference strip cut out, concentrate at one edge, elute finally with 1.5 ml of 0.0075 N HCl of which 100 µl are used for counting and 700 µl for determination of deoxycytidine. In the case of rat, deoxycytidine, present in much greater concentrations can be determined directly in 5 µl of fraction B 1 to which 0.7 ml of 0.0075 N HCl are added. Ten µl of fraction C is used for counting radioactivity.

Column chromatography of biogenic amines. Spike a 10 ml urine sample with 2 µg of histamine, dopamine, noradrenalin and serotonin (200 µl of 10 µg/ml solutions) and with 100 µg of spermidine (100 µl of 1 mg/ml solution). Apply 10 ml urine samples to Biorex 70 columns, wash with 30 ml of water containing 0.1 g EDTA/1 pH 7.0, then with 8 ml of 0.2 N HCl containing 0.1 g EDTA/1. Elute amines with 10 ml of 0.2 N HCl EDTA. Use 3×1 ml for serotonin, 2×1 ml for histamine spermidine. Neutralize 2 ml for determination of noradrenalin, dopamine (see under special methods). Hydrochloric acid blanks used in these assays must also pass the Biorex 70 column.

Chromatography of N-methyl pyridone carboxamide on layered column. Apply 750 µl urine and wash with 2 ml water; collect and pass again through column. Then wash with 3 ml water; collect total eluate and drain. Prepare also a column for blank.

Individual determinations

All determinations except where indicated otherwise (deoxycytidine) are made in duplicate. ΔE signifies that a respective photometric, ΔF that a fluorometric blank is subtracted. Δ Est and Δ Fst are the values for the standards indicated for the determinations. All calculations yield values in mg/ml urine (or μmole/ml). They must be divided subsequently by the creatinine concentration.

Na^+, K^+ ions. Dilute 0.5 ml of urine with 4.5 ml of water. Measure ion activity with specific electrodes (Orion), then spike with 100 μl 1 M KCl and 100 μl 1 M NaCl solution. Measure again. Calculate by known addition method.

Total and free sulfate (standard: 50 μl of 1 mg/ml K_2SO_4).

Total sulfate	*Free sulfate*
50 μl urine	50 μl urine
50 μl 2N HCl	50 μl 2N HCl
Hydrolyse in closed tube at 100°C for 4 hours	Continue directly
Add 2.5 ml of 5 % trichloracetic acid, centrifuge, remove	20 μl
1 ml	1 ml
200 μl reagent	200 μl

Read at 500 nm after 20 min.

$$\text{Total (or free) sulfate } \mu\text{mole/ml} = 28.7 \frac{\Delta \text{ E}}{\Delta \text{Est}}$$

Reagent: Dissolve 0.5 g gelatine Difco in 100 ml warm water, store overnight in refrigerator, dissolve 0.5 g Ba Cl_2 and store for 4 more hours. The reagent is stable in the refrigerator for 1 month. (DODGSON 1961).

Creatinine (standard: 10μl of 1 mg/ml 1 N HCl).

10 μl urine

1 ml alkaline picrate, freshly prepared by mixing 5 ml 1 N NaOH and 10 ml 0.6 % picric acid.

Read at 530 nm after 20 min standing.

$$\text{Creatinine mg/ml} = \frac{\Delta \text{E}}{\Delta \text{Est}}$$

Creatine (standards: 10 μl of creatine 1 mg/ml, 200 μl of creatinine 1 mg/ml).

The entire procedure is carried out in an ice bath, blanks are made for each urine sample and are measured immediately after addition of the reagents.

Both sets of standards, creatine and creatinine, must be run simultaneously.
50 μl urine (for rat use 10 μl)
1 ml freshly prepared mixture of 30 mg of α-naphthol in 10 ml of 0.5 N NaOH to which 1.5 ml of 0.04 % diacetyl in H_2O is added.
read at 530 nm the blank immediately, the assay after 30 min.

$$\text{Creatine (man) } \mu\text{g/ml} = \frac{200}{\Delta\text{Est-in}} \left[\Delta E - \left(\frac{\text{Concinin} \times \text{Estinin}}{4}\right)\right]$$

$$\text{Creatinine (rat) } \mu\text{g/ml} = \frac{1000}{\Delta\text{Est-in}} \left[\Delta E - \left(\frac{\text{Concinin} \times \text{Estinin}}{20}\right)\right]$$

Estin and Estinin are the standards for creatine and creatinine and Concinin the concentration of creatinine in mg/ml obtained for creatine in the test above (GERBER et coll. 1961).

Total amino acids and taurine (standards: 10 μl of 1 mg/ml taurine, 10 μl of 1 mg/ml alanine or 112 nmole)

Total amino acids		*Taurine*
5 μl urine		20 μl fraction A
1 ml	Ninhydrine freshly mixed	1 ml

Heat to 95°C for 20 min, cool and dilute with 4 ml of 50 % ethanol.
Read at 570 nm.

$$\text{Taurine } \mu\text{g/ml} = 1000 \frac{\Delta E}{\Delta\text{Est-taur.}}$$

$$\text{Total amino acids } \mu\text{mole/ml} = 22.4 \frac{\Delta E}{\Delta\text{Est-Al.}}$$

Ninhydrine: 25 ml 3 % ninhydrine in methylcellosolve
25 ml Citrate buffer (42 g citric acid, 22.4 g NaOH/1) pH 5.7
200 μl $SnCl_2$ (4.51 g $SnCl_2$ dissolved in 1.7 ml conc. HCl add 8.3 ml H_2O) (SØRBO 1961).

Delta amino levulinic acid (standard 10 μl of 100 μg/ml)

Sample		*Blank*
20 μl	fraction B	20 μl
10 μl	acetylacetone	0
500 μl	buffer pH 4.6	500 μl
	acetate buffer pH 4.6	

	(136 g Na acetate 57 ml acet. acid/l) heat 10 min to 95°C	
	acetylacetone	10 μl
500 μl	Ehrlich reagent (1 g dimethylaminobenzaldehyde in 34 ml acetic acid, add 16 ml $HClO_4$ conc.)	500 μl

Read at 546 nm after 15 min.

$$\text{Delta amino levulinic acid } \mu\text{g/ml} = 2.72 \times \frac{\Delta E}{\Delta Est}$$

(Sun et coll. 1969).

Total hydroxyproline (standard 10 μl of 100 μg/ml)
100 μl urine
100 μl HCl conc.
Hydrolyse at 100° in closed tubes for 18 hours
Neutralize with 1 N NaOH, adjust volume to 4 ml
500 μl sample
300 μl chloramin T freshly prepared solution
(1.41 g chloramin T/10 ml buffer pH 6.0 (50 g citric acid 12 ml acetic acid 120 g Na acetat 34 g NaOH 1250 ml H_2O and 250 ml n-propanol).
keep 20 min at 20°C add 300 μl Ehrlich reagent freshly prepared (1.5 g dimethylaminobenzaldehyde) in 7.4 ml n-propanol, add slowly 2.6 ml conc. perchloric acid).
Heat to 60° C for 15 min
Read at 555 nm

$$\text{Hydroxyproline } \mu\text{g/ml} = 80 \frac{\Delta E}{\Delta Est}$$

(Stegemann & Stalder 1967).

Hippuric acid (standard 5 μl of 500 μg/ml)
5 μl urine
3 ml H_2SO_4 70 % (3 parts H_2O add 7 parts H_2SO_4 Suprapur conc).
Use quartz cuvettes
Read in spectrofluorometer excitation at 260 nm, emission 375 nm

$$\text{Hippuric acid } \mu\text{g/ml} = \frac{\Delta F \times 500}{\Delta Fst}$$

(Ellman et coll. 1961).

Pseudouridine

1.2 ml eluate	1.2 ml eluate (from paperchromatogram)
100 μl 1N HCl	100 μl 1N NaOH

Read E at 290 nm
Calculate ΔE = E290 alk − E290 acid
Count 300 μl by liquid scintillation counting

$$\text{Pseudouridine } \mu g/ml = 0.00068 \times \Delta E \times \frac{\text{Act added}}{\text{Act sample}}$$

Uric acid

Use kit marketed by Boehringer (Germany)
50 μl urine
8 ml borate buffer pH.9.5 (sol 1)
mix remove for:

Assay		*Blank*
3.5 ml	urine dilution	3.5 ml
20 μl	uricase (sol 2)	0
0	50 % glycerol (sol 3)	20 μl

Mix, measure after 10 min at 293 nm
Uric acid $\mu g/ml = \Delta E \times 2.15$

Deoxycytidine

700 μl in 0.0075 N HCl (eluate from paper (man) or diluted fraction (rat) heat to 95°C for 2 hours, add
100 μl of 0.025 M periodate in 0.125 N H_2SO_4 (535 mg Na periodate 150 μl H_2SO_4 conc/100 ml H_2O)
20 min at room temperature
200 μl Na arsenite 2 % in 0.5 N HCl
400 μl Thiobarbituric acid (0.75 g in 100 ml H_2O add 700 μl 1 N NaOH) heat to 95° C for 20 min
cool, extract with 1.2 ml cyclohexanone, centrifuge
read at 532 and 562 nm.
ΔE = 'E 532 − E 562

$$\text{Deoxycytidine (man) } \mu g/ml \text{ urine} = 0.0143 \frac{\Delta E \times \text{Act added}}{\Delta \text{Est} \times \text{Act sample}}$$

$$\text{Deoxycytidine (rat)} = 0.2\ \frac{\Delta E \times \text{Act added}}{\Delta \text{Est} \times \text{Act sample}}$$

(CHEN et coll. 1968).

Kynurenic acid, Xanthurenic acid (standard 10 μl of 100 μg/ml)

Kynurenic acid;		*Xanthurenic acid*
200 μl fraction B		200 μl fraction B
1.8 ml H_2O		1.3 ml H_2O
cool in ice 1 ml H_2SO_4 conc. Suprapur		1.5 ml 40 % NaOH
Read in fluorometer after 30 min.		
Excitation	345 nm	345 nm
Emission	440 nm	420 nm

$$\text{Kynurenic (Xanthurenic acid) } \mu\text{g/ml} = \frac{\Delta F \times 5}{\Delta \text{Fst}}$$

(PRICE et coll. 1965).

N-Methyl-nicotinamide (standard 10 μl of 100 μg/ml)

50 μl fraction C
50 μl methylethylketone Mn Cl_2 (2 ml/l methylethylketone of 0.1 M (1.25 %) $MnCl_2$ solution)
50 μl 5N NaOH
shake, wait 10 min
50 μl 6 N HCl prepare blanks without methylethylketone
heat 10 min to 95° C add
2.5 ml 2.5 % KH_2PO_4

Spectrofluorometer	Excitation	360 nm
	Emission	450 nm

$$\text{N-Methyl-nicotinamide } \mu\text{g/ml} = \frac{\Delta F \times 3.7}{\Delta \text{Fst}}$$

(PRICE et coll.)

Indoxylsulfate (Indicane) (standard 10 μl of 1 mg/ml)

500 μl fraction A (rat 100 μl)
500 μl Ehrlich reagent freshly prepared (0.89 dimethylaminobenzaldehyde dissolved in 30 ml methanol+30 ml HCl conc.)
20 min
read at 480 mμ

$$\text{Indoxylsulfate (man) } \mu g/ml = \frac{40\ \Delta E}{\Delta Est}$$

$$\text{Indoxylsulfate (rat) } \mu g/ml = \frac{200\ \Delta E}{\Delta Est}$$

(RYLANCE 1969).

N-Methyl-2-pyridone 5 carboxamide
Read effluent from column at 310 nm.
Compare with effluent of blank column.
N-Methyl-2-pyridone-5-carboxamide $\mu g/ml = 0.389 \times \Delta E310$
(PRICE et coll.)

Sialic Acid total (standard 10 μl of 100 $\mu g/ml$)
50 μl urine
50 μl H_2SO_4 0.5 N (1 part H_2SO_4 conc./64.7 parts H_2O)
heat 1 hour at 80° C, add
100 μl 0.2M perjodate (8.556 g in 200 ml 4.5 M H_3PO_4 (1 part conc./3.37 parts H_2O)
20 min at 20° C then add
500 μl arsenite (10 % Na arsenite in 0.5M Na_2SO_4 (71 g/l Na_2SO_4) plus 60 ml/l H_2SO_4 conc.)
shake until brown color disappears (heat slightly if necessary) add 1.5 ml thiobarbituric acid (0.6 % in Na_2SO_4 0.5M (71 g/l))
heat to 95° for 10 min, cool, add
2 ml cyclohexanone, shake, centrifuge
read E of upper layer at 545 nm (wash cuvettes before and after with methanol).

$$\text{Total sialic acid } \mu g/ml = \frac{\Delta E \times 20}{\Delta Est}$$

(WARREN 1959).

Noradrenalin/Dopamine (standard 200 μl of 10 $\mu g/ml$ diluted in 10 ml 0.2 N HCl EDTA and neutralized as described below)
2 ml eluate from Biorex 70 column
1 ml phosphate buffer pH 7.0 (4.27 g Na_2HPO_4 2 H_2O, 9.25 g KH_2PO_4 0.2 g EDTA/200 ml. This is the same buffer as used for preparation of resin but 5 times concentrated
200 μl 1N NaOH to adjust pH at 6.5 to 7; check with pH meter

500 μl H_2O to adjust volume to 4 ml, then divide into
2 ml samples.
200 μl iodine solution (2 g KI 0.5 g iodine /40 ml H_2O)
mix well and wait exactly 2 min.
500 μl freshly prepared sulfite (1 ml 25 % Na_2SO_3 plus 9 ml 5N NaOH)
wait 2 min then add
400 μl conc. acetic acid
heat to 45°C for 3 min.
Read noradrenalin in spectrofluorometer
excitation 400 nm
emission 485 nm
heat again to 85°C for 30 min.
Read dopamine in spectrofluorometer using quartz cuvettes.
excitation 330 nm
emission 400 nm

$$\text{Noradrenalin-Dopamine } \mu\text{g/ml} = \frac{0.2\ \Delta F}{\Delta Fst}$$

(CHANG 1964).

Histamine-Spermidine (free) (standards 200 μl of 10 μg/ml histamine, 100 μl of 1 mg/ml spermidine diluted separately in 10 ml 0.2 N HCl EDTA)
1 ml eluate from Biorex 70 column
500 μl 5 N NaOH
100 μl 0.1 % o-phtaldialdehyde in methanol
mix well, wait 4 min, add
800 μl 4M H_3PO_4 (28 ml conc. H_3PO_4 plus 72 ml H_2O)
read in spectrofluorometer after 30 min
excitation 360 nm
emission 405 and 500 nm

$$\text{Histamine } \mu\text{g/ml} = 0.2 \times \frac{(\Delta F_{500} \times \Delta F_{sp405}) - (\Delta F_{405} \times \Delta F_{sp500})}{(\Delta F_{H500} \times \Delta F_{sp405}) - (\Delta F_{H405} \times \Delta F_{sp500})}$$

$$\text{Spermidine } \mu\text{g/ml} = 10 \times \frac{(\Delta F_{405} \times \Delta F_{H500}) - (\Delta F_{500} \times \Delta F_{H405})}{(\Delta F_{H500} \times \Delta F_{sp405}) - (\Delta F_{H405} \times \Delta F_{sp500})}$$

where F405, F500 are the readings of the sample at the two wavelengths after substraction of blank fluorescence
F_{sp405}, F_{sp500} the readings of the spermidine standard
F_{H500}, F_{H405} the readings of the histamine standard
(OATES et coll. 1962).

Serotonin (standard 200 µl of 10 µg/ml diluted with 10 ml 0.2 HCl EDTA)

Sample (2×)		*Blank* (1×)
1.6 ml	HCl conc.	1.6 ml
100 µl	cysteine 1 % in 0.1 N HCl	
	$NaIO_4$ 0.02 % in 0.1 N HCl	100 µl
1 ml	eluate Biorex 70	1 ml
Mix, incubate at 20°C 30 min		
100 µl	$NaIO_4$	Ø
Ø	cysteine	100 µl
100 µl	0.1 % o-phthalaldehyde in methanol	100 µl

mix, heat to 95°C for 15 min, cool
read in spectrofluorometer excitation 360 nm
emission 480 nm

$$\text{Serotonin } \mu g/ml = \frac{0.2\ \Delta F}{\Delta Fst}$$

(MAICKEL et coll. 1969).

5-hydroxyindole acetic acid (standard: direct 10 µl and extraction 50 µl of 50 µg/ml, respectively)
300 µl urine
300 µl H_2O
0.6 g NaCl
50 µl 4 % cysteine
180 µl HCl conc.
12 ml diethylether
extract by shaking for 1 min. Remove from upper phase
9 ml ether, add
4 ml phosphate buffer pH 7.0 (3.54 g $K H_2PO_4$ $2H_2O$, 7.24 g $Na_2H PO_4$ $2 H_2O$) shake 1 min centrifuge, remove 3 samples of 1 ml
proceed for determination as described for serotonin, replacing eluate with the phosphate buffer extract.

$$\text{5-OH-indoleacetate } \mu g/ml = 8.33 \frac{\Delta F}{\Delta Fst \text{ extern}} \text{ or } \frac{8.88 \Delta F}{\Delta Fst \text{ direct}}$$

(KORF & VALKENBURGH-SIKKEMA 1969).

Proteolytic activity (cathepsin D, E.C. 3.4.4,23)
50 µl urine

200 μl formate buffer 0.2 N pH 3.5 (11.2 ml formic acid/500 ml adjusted with NaOH to pH. 3.5 and filled up to 1 l)
500 μl hemoglobin (2.5 % in 0.003N H Cl) (prepared from erythrocytes)
Incubate at 45°C for 1 hour add
1 ml 5 % trichloracetic acid,
centrifuge, remove from supernatant
500 μl, add
300 μl Folin reagent (Folin, Merck, diluted 1/3 with H_2O)
1 ml 1 N NaOH
read after 10 min at 600 nm
prepare also blank by adding TCA before incubation
Standard 10 nmole tyrosine (10 μl of 182 μg/ml)
1 mU = liberation of 1 nmole tyrosine/min/ml urine

$$\text{Proteolytic activity mU/ml} = 21 \times \frac{\Delta E}{Est}$$

(BARRETT 1967).

Amylase (E.C.3.2.1.1.) (Use Rapidstat Kit from Pierce Chem Co) (Standard 10 μl of 2 mg/ml solution of glucose)

Test	*Blank*
10 μl urine (diluted rats 1/10 with saline)	10 μl urine
100 μl substrate	100 μl color reagent
Incubate 15 min at 37°C	
100 μl color reagent	100 μl substrate
Heat 10 min to 95°C	
Cool 1 min, add	
800 μl H_2O	800 μl H_2O

read at 500 nm
1 mU = liberation of μmole glucose/min/ml urine

$$\text{Amylase (man) mU/ml} = 0.747 \times \frac{\Delta E}{Est}$$

$$\text{Amylase (rat) mU/ml} = 7.47 \times \frac{\Delta E}{Est}$$

Ribonuclease (E.C.2.7.7.16)
20 μl urine
400 μl buffer substrate freshly prepared by mixing 2 parts Tris buffer 0.1M

pH 7.4 (12.1 g Tris 10 ml 1 N HCl/1) and 1 part 0.8 % RNA dialyzed previously for 3 hours against distilled water.
Incubate at 37°C for 1 hour, prepare also blanks without incubation and without substrate, add
200 μl uranylacetate 0.75 % in 4.17 M perchloric acid (1 part $HClO_4$ conc., 2 parts H_2O)
centrifuge, remove
200 μl supernatant, dilute with
5 ml H_2O

read E at 260 nm $\qquad 1\ mU = \frac{\Delta E_{260}}{min\ ml}$

RNA ase U/ml = $0.0125 \times \Delta E$
(KALNITZKY et coll. 1959).

β-Glucuronidase
50 μl urine
100 μl buffer substrate (10.32 mg phenolphthalein glucuronide in 10 ml acetate buffer pH 4.5 (3.4 g Na acetate, 1.54 ml acetic acid /500 ml)
incubate at 37°C for 6 hours (man) or 1 hour (rat) add
1 ml glycine NaOH (18 g glycine 1 N NaOH to pH 10.5/1)
Read at 546 nm. Prepare also a few blanks without incubation
Glucuronidase (man) mU/ml = $0.0025 \times \Delta E$ (1 mU = 1 μmole phenolphtaleine liberated/min)
Glucuronidase (rat) mU/ml = $0.0151 \times \Delta E$
(FISHMAN 1965).

SUMMARY

Since the development of biochemical indicators of radiation injury has been hampered often by the lack of simple and rapid methods of determination, techniques for the measurement of 30 urinary compounds are presented. These methods have been in part taken from the literature, in part they have been modified or developed by us. They represent a compromise between speed and simplicity of execution and specificity of assay.

ZUSAMMENFASSUNG

Die Entwicklung biochemischer Indikatoren des Strahlenschadens leidet unter dem Fehlen einfacher und rascher Bestimmungsmethoden. In dieser Arbeit sind solche Methoden für 30 verschiedene, in den Urin ausgeschiedene Stoffe zusammengefasst. Sie wurden teils der Literatur entnommen, teils in unserm Labor modifiziert oder entwickelt. Hierbei wurde nach einem Kompromiss zwischen Einfachheit und Schnelligkeit der Durchführung und Spezifität der Analyse gesucht.

RÉSUMÉ

Vu que développement des indicateurs biochimiques de lésions des radiations a souvent été entravé par le manque de méthodes de détermination simples et rapides, des techniques de mesures de 30 composés urinaires sont présentées. Ces méthodes ont été puisées en partie dans la littérature que nous avons modifiées ou développées. Elles représentent un compromis entre la rapidité, la simplicité de l'exécution et la spécificité de l'analyse.

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