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(54) Titre : FIXATIF DE TISSUS ET MODE D'UTILISATION
(54) Title: TISSUE FIXATIVE AND METHOD

(57) Abrégé/Abstract:
Tissue fixatives, such as diazolidinyl urea, which are free of aldehydes and toxic chemicals are described. When used, either in aqueous or alcoholic solutions, good tissue preservation is attained. In addition, tissue antigens are retained which makes the fixative useful for immunostaining procedures.

TISSUE FIXATIVE AND METHOD

ABSTRACT OF THE DISCLOSURE

Tissue fixatives, such as diazolidinyl urea, which are free of aldehydes and toxic chemicals are described. When used, either in aqueous or alcoholic solutions, good tissue preservation is attained. In addition, tissue antigens are retained which makes the fixative useful for immunostaining procedures.

TISSUE FIXATIVE AND METHOD

BACKGROUND OF THE INVENTION

The present invention relates to compositions for the fixation of cells and tissues and to methods for the fixation of cells and tissues using as the fixing agents certain compounds.

The objective of tissue fixation is to provide as much detail of the cell as possible. To do this, it is necessary to maintain the cells in their original unaltered morphology so that maximum cellular detail is observed under the microscope. With the development of immunostaining there is also the requirement that the antigens of the cells are not altered by the method of fixation or stabilization. Although the microscope is the usual means for examining cells that are fixed and stained, they may also be examined by the laser or the flow cytometer. The flow cytometer is an important method for examining a large number of cells in a brief time.

The usual formulations for stabilization of cells contain one or more agents which react vigorously with the proteins of the cells to denature, coagulate and insolubilize the components of the cell. Typical of this type of agent is picric acid, mercuric ions, formaldehyde and glutaraldehyde. In addition, some less toxic compounds which can also denature and stabilize the proteins are acetic and formic acid, but these are often less suitable for a number of histological procedures.

Unfortunately, the toxicity associated with these compounds renders their use less than satisfactory. For example, formaldehyde, the most common of these

fixatives, is a noxious gas which is also toxic, flammable and carcinogenic. Although efforts are made when this chemical is used to protect workers and avoid contamination of the drainage system when disposed, these efforts are usually both expensive and inconvenient, and fixatives such as formaldehyde still present a danger to laboratory workers and health care professionals. It is thus highly desirable to develop fixatives which can be used safely, effectively and conveniently in histological studies.

OBJECTS OF THE INVENTION

Thus, it is an object of the invention to provide a fixative solution for tissues and cells which has an extremely low toxicity yet meets all of the requirements of a model fixative.

Another object of the invention is to provide a fixative solution for tissues and cells that preserves tissues and cells and their cellular detail.

Another object of the present invention is to provide a fixative which in addition to being low in toxicity gives off no noxious fumes, is not flammable or carcinogenic, and which can be disposed of safely and conveniently when use is completed.

Yet another object of the invention is to provide a fixative solution for tissues and cells that preserves tissues and cells and their antigenic detail to allow for the satisfactory conducting of immunohistochemical and other immunological techniques on the tissues and cells.

Yet another object of the invention is to provide a fixative solution that provides an unaltered antigenic surface for reaction with specific antibodies.

SUMMARY OF THE INVENTION

These and other objects of the invention are obtained by a fixative solution for tissues and cells comprising histological fixing amounts of at least one active agent selected from the group consisting of:

i) diazolidinyl urea

ii) imidazolidinyl urea

iii) dimethylol-5,5-dimethylhydantoin

iv) dimethylol urea

v) 2-bromo-2-nitropropane-1,3-diol; and

vi) quaternary adamantane (e.g. 1-(3-chloroallyl)-3,5,7-tri-aza-1-azoiadamantiane-chloride, N-(3-chloroallyl)-hexammonium chloride such as Dowicil 200; Dowicide Q; Preventol D1)

in a solvent selected from water, dimethylsulfoxide and an alcohol and mixtures thereof.

In another aspect, the invention comprises an improvement in a method of fixing tissues and cells with a histological fixative wherein the histological fixative is an active agent selected from at least one of the group consisting of:

i) diazolindinyl urea

ii) imidazolidinyl urea

iii) dimethylol-5,5-dimethylhydantoin

iv) dimethylol urea

v) 2-bromo-2-nitropropane-1,3-diol; and

vi) quaternary adamantane.

A preferred fixative solution for tissues and cells of the invention comprises histological fixing amounts of the following ingredients:

i) diazolidinyl urea;

ii) 2-bromo-2-nitropropane-1,3-diol (also known as "Bronopol"); and

iii) a water-soluble zinc salt

in a solvent selected from the group consisting of water, alcohol, dimethylsulfoxide and mixtures thereof. In the preferred embodiment of this solution, the water-soluble zinc salt comprises zinc sulfate. If desired, the above fixative solution can be buffered to a pH of about 4-6 through the addition of a suitable buffer such as a citrate buffer.

In another aspect of the preferred embodiment, the invention comprises an improvement in a method of fixing tissues and cells with a histological fixative wherein the histological fixative is an active agent consisting of:

i) diazolidinyl urea;

ii) 2-bromo-2-nitropropane-1,3-diol (also known as "Bronopol"); and

iii) a water-soluble zinc salt.

Unlike the typical histological fixing agents, the active agents of the invention have extremely low toxicity. For example, toxicity studies comparing diazolidinyl urea of the invention with formaldehyde of the prior art show the following:

	Inhalation Toxicity	Dermal Toxicity	LD 50
Formaldehyde	500 mg/Kg	270 mg/Kg	800 mg/Kg
Diazolidinyl urea	None	2000 mg/Kg	2570 mg/Kg

This reduced toxicity makes disposal and handling less of a problem. In addition, since there is no inhalation toxicity, there are no badge detection devices required as there are for formaldehyde.

Another advantage offered by the active agents of the invention is the fact that they are not flammable and therefore do not present a fire hazard as do many of the prior art fixatives.

The mechanism by which the active agents of the invention provide the desired tissue and cell membrane is not known for certain. It is believed that the active agent binds in some fashion to the cell membrane or tissue to stabilize. This hypothesis is drawn because many of the active agents of the invention are known disinfectants which kill bacteria by binding to cell structures. This is not a full explanation of the mechanism responsible for the results of the invention since many other disinfectants such as Kathon* and Omadine* fail to provide tissue and cell stabilizing effects.

The ability of the active agents of the invention to preserve antigens is also not understood but it is probably due to a difference in the reaction between the active agents of the invention and prior art fixatives such as formaldehyde with proteins. Formaldehyde cross-links with itself and proteins to obscure the antigen. To determine if this is true, diazolidinyl urea was added to the protein albumin to stabilize it. After incubation of diazolidinyl urea and protein mixture for 24 hours, disc-gel electrophoresis indicated no change in the rate of migration of the protein. When this experiment is conducted with formaldehyde, a large number of multimers and insoluble protein results.

* Trade-mark

In another aspect of the invention, it has been found that the addition of alkali metal salts of ascorbic acid increases the activity of the active agents of the invention in fixing the tissue or cell membrane.

DETAILED DESCRIPTION OF THE INVENTION

The fixative solutions of the invention are comprised of the active agents in solvent selected from water, dimethylsulfoxide alcohol and mixtures thereof.

The alcohol solvent comprises one or more alkanols such as methanol, ethanol, propanol and butanol; polyols, e.g. diols or triols such as ethylene glycol, glycerol, propylene glycol and trimethylene glycol and mixtures of alkanols and polyols. It is also preferable that a suitable buffer such as a citrate buffer be added to the solution to adjust the pH to about 4-6. One particularly preferred citrate buffer to be used in the solution is sodium citrate dihydrate, but other buffers can be used as would be obvious to one skilled in the art.

Whether the solvent employed is water, alcohol solvent, dimethylsulfoxide or a mixture thereof depends principally upon the tissue or membrane being fixed. For example, where large pieces of tissue are being fixed, it is preferred to use an alcohol solvent or aqueous alcohol solvent since the alcohol solvents increase penetration. Also, in fixing cells such as Pap smears, the alcoholic preparations are preferred because they cause the cells to stick to the slides. When aqueous alcoholic solutions are employed as the solvent for the active agents of the invention, the ratio of alcohol to water will fall in the range of 4:1 to 2:1.

The amount of the active agents in the formulation of the invention is that effective to fix or stabilize the tissue or cell membrane. Generally, this amount falls in the range of about 20 to 100 grams per liter, preferably 50 to 75 grams per liter.

Generally, in the preferred embodiment the compositions are comprised of about 20-40 grams of Bronopol (about 30 grams particularly preferred), 20-40 grams of diazolidinyl urea (about 30 grams particularly preferred) and about 10-15 grams of the water-soluble zinc salt (about 12 grams particularly preferred) per 1000 ml of solvent used. It is preferred that zinc sulfate, and more particularly zinc sulfate heptahydrate, be employed as the water-soluble zinc salt, but a number of other zinc salts will also be suitable as would be evident to one of ordinary skill in the art. For example, zinc salts such as zinc chloride or zinc acetate could also be employed, but these are considered less effective than zinc sulfate. In addition to the zinc salt, it is preferable to add about 2-6 grams of a citrate buffer (about 3 grams particularly preferred) such as sodium citrate dihydrate to the above fixative solution.

In terms of percentages, it is preferred that the fixative solution comprise about 1-5% Bronopol (about 3% particularly preferred) and about 1-6% diazolidinyl urea (about 3% particularly preferred). In the preferred embodiment, about 0.02 to 0.1 g-mol/L zinc salt (about 0.05 particularly preferred) is added to the fixative solution along with the Bronopol and diazolidinyl urea.

When alkali metal ascorbic acid salts such as sodium ascorbate are included to increase the activity of the active agents to fix the tissue or cells, they

are added in an amount of about .25 to 1 grams per liter.

The solute in the preparations of the invention may also include any of the other addendum conventionally added to histological fixative preparations. These addendum include mordants, buffers, penetration increasers, osmotically active substances and nuclear detail improvers and nuclear size increasers.

Examples of suitable mordants are salts with a metal ion having an oxidation state of two or more. Illustrative are zinc, strontium, calcium, barium and chromium salts. The preferred salt is zinc sulfate.

Suitable buffers include alkali metal phosphate salts such as sodium phosphate and potassium phosphate.

Osmotically active substances that may be included in the formulation of the invention are alkali metal salts such as sodium chloride. In addition, sugars such as the polysaccharides, sucrose, glucose and the like may be employed.

Nuclear detail improvers and nuclear size increasers include acetic acid and lithium salts such as lithium chloride. Zinc salts such as zinc sulfate not only improve nuclear definition but also improve staining.

Illustrative of substances which increase the rate of penetration of the fixing agent are dimethylsulfoxide and ethanol.

In the preferred embodiment, the active fixative ingredients described above are dissolved in a suitable solvent such as distilled water, and this solution can then be used as a fixative agent in a number of ways as would be obvious to one skilled in the art. For example, the fixative solution can be

used to preserve samples of tissue that are being shipped or carried to an examination site. In this process, small vials or jars that have liquid tight seals are filled with the reagent of the invention, and tissue samples are placed in the reagent-containing vial to preserve the samples until they reach an area where further processing can occur.

Tissues prepared for study using the fixative of the invention can be prepared for histological study in any known conventional manner, such as through the use of paraffin, sectioning equipment, staining, mounting on slides, or other common steps utilized prior to microscopic or other examination. The present invention thus provides a safe, convenient and effective fixative solution which can be utilized in the many known histological procedures that employ such solutions.

The following examples are illustrative of formulations of the invention.

EXAMPLE I

Diazolidinyl urea	50 g/L
Na_2HPO_4	0.73 g/L
$KHPO_4$	0.02 g/L
NaCl	8.50 g/L
Distilled H_2O to one liter	

EXAMPLE II

Diazolidinyl urea	50 g/L
Ethanol	500 ml
Acetic acid, conc.	10 ml
Distilled H_2O to one liter	

EXAMPLE III

Diazolidinyl urea	50 g/L
Lithium chloride	6.35 g/L
Distilled H_2O to one liter	

EXAMPLE IV

Diazolidinyl urea	50 g/L
Dimethylsulfoxide	100 ml
Distilled H_2O to one liter	

EXAMPLE V

Diazolidinyl urea	50 g/L
Dimethylsulfoxide	100 ml
Zinc chloride	5.8 g/L
Distilled H_2O to one liter	

EXAMPLE VI

Diazolidinyl urea	50 g/L
Ascorbic acid, sodium	.25 g/L
Distilled H_2O to one liter	

The following is an example of the use of fixatives of the invention.

EXAMPLE VII

Tissue is immersed in the fixative of Example I for four hours. The treated tissue is then dehydrated through a series of graded alcohols, cleared in xylene and impregnated with molten paraffin. This procedure is performed under heat and vacuum/pressure in a 12-hour cycle using a Fisher Histomatic* (Model 166 MP) tissue processor. The tissue is then blocked, paraffin embedded, rehydrated in ice water for a minimum of three hours to enhance sectioning, and sectioned at 4-5 microns. The tissue is mounted on a glass slide, deparaffinized, stained, coverslipped and evaluated microscopically.

The following example demonstrates the satisfactory results obtained with the fixative of the invention using various staining methods.

EXAMPLE VIII

* Trade-mark

Example VII is repeated using the staining method identified. The results in each case are as follows:

Staining Method	Results
Mayer's mucicarmine	Demonstrable; well-defined
Elastin	Satisfactory detail
Movat's reticulin stain	Satisfactory detail; minimal shrinkage
Gomori's trichrome stain	Fibrous tissue; well-defined
Periodic Acid-Schiff (PAS)	Non-specific staining not evidenced as in formalin-fixed preparation
Geimsa	Satisfactory detail
Hematoxylineosin & Eosin (H&E)	Satisfactory detail

The following example demonstrates the ability of the fixative of the invention in retaining tissue antigens in immunostaining procedures.

EXAMPLE IX

The tissues identified below having the antigenic sites identified below are fixed with the fixative formulation of Example I and immunohistochemically stained using avidin-biotin stainings.

Tissue	Markers Detected
Lymph node	LN-1 LN-2 LN-3 UCA L-26

	LCHL-1
Brain	Neurofilament Glial Fibrillary Acidic Protein
Hodgkins node	Ber H_2 Leu M_1
Colon	Cytokeratin MAK-6 Cytokeratin AE1/AE3
Muscle	Desmin
Pituitary	S-100
Thyroid	Thyroglobulin
Breast	α-lactalbumin

None of the antigenic sites are affected by the immunostaining.

EXAMPLE X

A fixative in accordance with the present invention was prepared having the following formulation:

30 grams of Bronopol

30 grams of Diazolidinyl urea

12 grams of zinc sulfate heptahydrate

2.9 grams of sodium citrate dihydrate

dissolved in 1000 ml distilled water.

This solution was used as a fixative for tissue samples by placing the samples in a vial containing the fixative solution, and holding the sample for about four hours in the fixative. After the tissue has been sufficiently treated with fixative, it is

then dehydrated using a series of graded alcohols, cleared in xylene and impregnated with molten paraffin. This procedure is performed under heat and vaccuum/pressure in a 12-hour cycle using a Fisher Histomatic Model 166MP tissue processor. The tissue is then blocked, paraffin embedded, rehydrated in ice water for about three hours to enhance sectioning, and sectioned at 4-5 microns. The tissue is mounted on a glass slide, deparaffinized, stained, coverslipped and evaluated microscopically.

Through use of the composition and method of the present invention, satisfactory results have been obtained with a variety of staining methods. The following results have been obtained using the fixative of the invention:

STAINING METHOD	RESULTS
Mayer's mucicarmine	Demonstrable; well-defined
Elastin	Satisfactory detail
Movat's reticulin stain	Satisfactory detail; minimal shrinkage
Gomori's trichrome stain	Fibrous tissue; well-defined
Periodic Acid-Schiff (PAS)	Non-specific staining not evidenced as in formalin-fixed preparation
Geimsa	Satisfactory detail

Hematoxylin & eosin (H&E) Satisfactory detail

EXAMPLE XI

The tissues identified below having the antigenic sites identified below are fixed with the fixative formulation of Example X and immunohistochemically stained using avidin-biotin stainings.

Tissue	Markers Detected
Lymph node	LN-1 LN-2 LN-3 LCA L-26 UCHL-1 B72.3
Brain	Neurofilament Glial Fibrillary Acidic Protein Vimentin
Hodgkins node	Ber H_2 Leu M_1
Colon	Cytokeratin MAK-6 Cytokeratin AE1/AE3 Epithelial Membrane Ag (EMA)
Muscle	Desmin Smooth Muscle Actin
Pituitary	S-100
Thyroid	Thyroglobulin
Breast	α-lactalbumin Estrogen Receptors (ERs) Progesterone Receptors (PRs)

Skin	HMB 45 Melanoma	

None of the antigenic sites are affected by the immunostaining.

CLAIMS:

1. A fixative solution for tissues and cells comprising histological fixing amounts of the following compounds:

i) diazolidinyl urea;

ii) 2-bromo-2-nitropropane-1,3-diol; and

iii) a water-soluble zinc salt in a solvent selected from the group consisting of water, alcohol, dimethyl-ulfoxide and mixtures thereof.

2. A fixative solution according to claim 1 further comprising a citrate buffer.

3. A fixative solution according to claim 2 wherein the citrate buffer comprises sodium citrate dihydrate.

4. A fixative solution according to any one of claims 1 to 3 wherein the water-soluble zinc salt is selected from the group consisting of zinc sulfate, zinc acetate and zinc chloride.

5. A fixative solution according to any one of claims 1 to 4 wherein the water-soluble zinc salt comprises zinc sulfate.

6. A fixative solution according to any one of claims 1 to 5 wherein the water-soluble zinc salt comprises zinc sulfate heptahydrate.

7. A fixative solution according to any one of claims 1 to 6 wherein the solution is buffered to a pH of about 4-6.

8. A fixative solution according to any one of claims 1 to 7 comprising about 1-5% w/v 2-bromo-2-nitropropane-1,3-

diol, about 1-6% diazolidinyl urea and about 0.02 to 0.1 g-mol./L zinc salt.

9. A fixative solution according to any one of claims 1 to 8 comprising about 3% w/v 2-bromo-2-nitropropane-1,3-diol, about 3% diazolidinyl urea and about 0.05 g-mol./L zinc salt.

10. A fixative solution according to any one of claims 1 to 9 further comprising a citrate buffer.

11. A fixative solution according to any one of claims 1 to 10 wherein the solvent is water.

12. A fixative solution according to any one of claims 1 to 10 wherein the solvent is alcohol.

13. A fixative solution according to any one of claims 1 to 12 comprising the following ingredients:

i) about 20-40 grams of diazolidinyl urea;

ii) about 20-40 grams of 2-bromo-2-nitropropane-1,3-diol; and

iii) about 10-15 grams of a zinc salt dissolved in about 1000 ml of distilled water.

14. A fixative solution according to claim 13 further comprising about 2-6 grams of a citrate buffer.

15. A fixative solution according to any one of claims 1 to 13 comprising the following ingredients:

i) about 30 grams of diazolidinyl urea;

ii) about 30 grams of 2-bromo-2-nitropropane-1,3-diol; and

iii) about 12 grams of a zinc salt dissolved in about 1000 ml of distilled water.

16. A fixative solution according to claim 15 further comprising about 3 grams of a citrate buffer.

17. In a method of fixing tissues or cells by treating same with a histological fixative, the improvement comprising employing as said histological fixative a composition comprising:

i) diazolidinyl urea;

ii) 2-bromo-2-nitropropane-1,3-diol; and

iii) a water-soluble zinc salt.

18. The method according to claim 17 wherein the composition further comprises a citrate buffer.

19. The method according to claim 18 wherein the citrate buffer comprises sodium citrate dihydrate.

20. The method according to any one of claims 17 to 19 wherein the water-soluble zinc salt is selected from the group consisting of zinc sulfate, zinc acetate and zinc chloride.

21. The method according to any one of claims 17 to 20 wherein the water soluble zinc salt comprises zinc sulfate.

22. The method according to any one of claims 17 to 21 wherein the zinc salt comprises zinc sulfate heptahydrate.

23. The method according to any one of claims 17 to 22 wherein the composition is dissolved in a solvent selected from water, an alcohol and mixtures thereof.

24. The method according to any one of claims 17 to 23 wherein the composition comprises about 1-5% w/v 2-bromo-2-nitropropane-1,3-diol, about 1-6% diazolidinyl urea and about 0.05 g-mol./L zinc salt.

25. The method according to claim 24 wherein the composition further comprises a citrate buffer.