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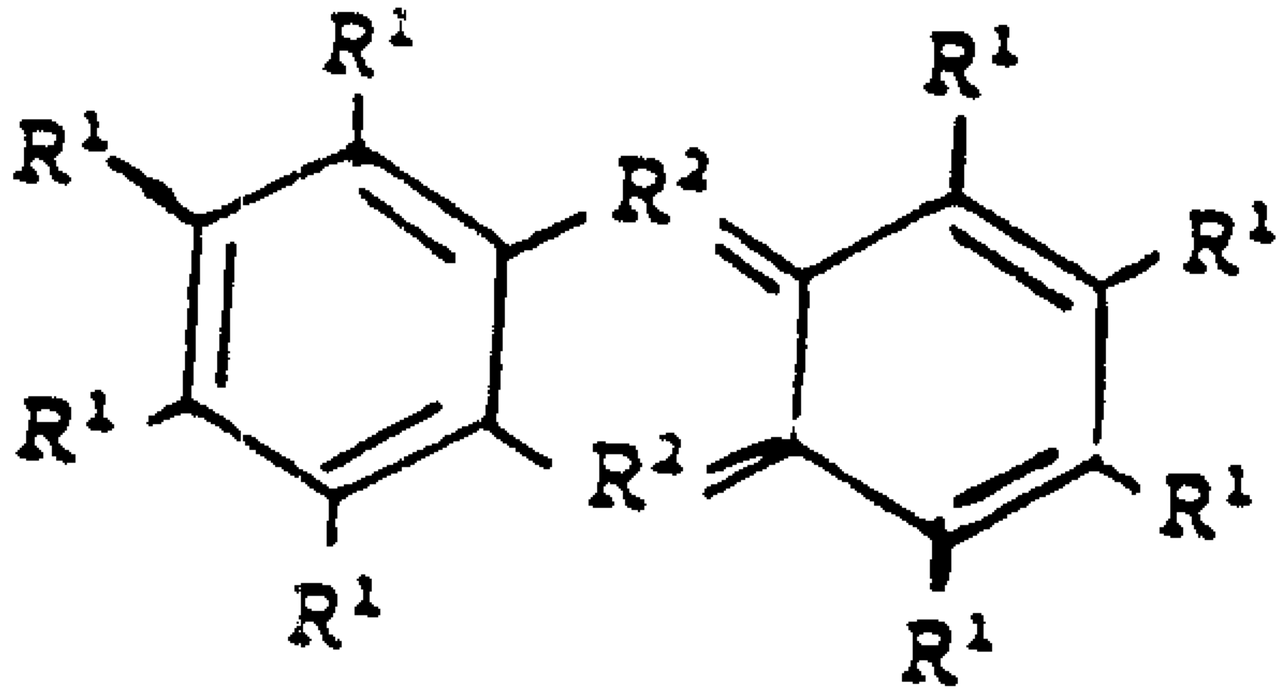
(54) Titre : PROCEDES ET COMPOSITIONS DE DETECTION IN-VIVO DES CANCERS ET DES ETATS DE PRE-CANCER DE LA CAVITE BUCCALE

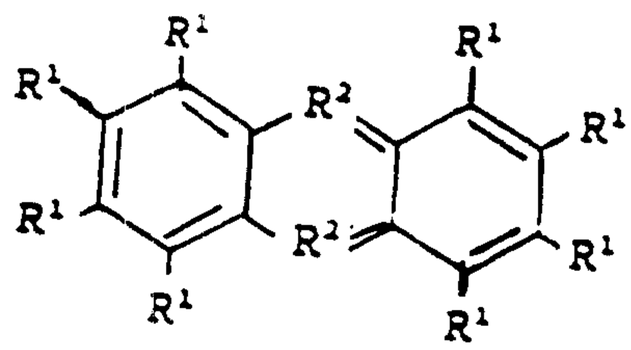
(54) Title: METHODS AND COMPOSITIONS FOR IN-VIVO DETECTION OF ORAL CANCERS AND PRECANCEROUS CONDITIONS



(57) Abrégé/Abstract:
A kit is provided for in vivo detection of cancerous and precancerous tissue. The kit includes two solutions. The first solution is a biological stain composition for rinsing the oral cavity, and comprises both a non-toxic dye other than toluidine blue O and methylene blue, the non-toxic dye having the structure (see above formula) and ionic derivatives thereof, wherein R^1 is H, a lower alkyl group or $N(R^3)_2$, R^2 is N, S or O, and R^3 is H or a lower alkyl group, and a pharmaceutically-acceptable oxidizing agent for leuco forms of the dye. The second solution is a rinse composition for removing unretained stain composition. The use which is taught for such kit includes the step of sequentially rinsing the oral cavity with that stain composition which is selectively retained by cancerous and precancerous oral tissues. The dye in the stain composition consists essentially of a dye which is either Azure B, Azure C or Brilliant Cresyl Blue. The oral cavity is then rinsed with a rinse composition for removing unretained stain composition.

ABSTRACT

A kit is provided for *in vivo* detection of cancerous and precancerous tissue. The kit includes two solutions. The first solution is a biological stain composition for rinsing the oral cavity, and comprises both a non-toxic dye other than toluidine blue O and methylene blue, the non-toxic dye having the structure



and ionic derivatives thereof, wherein R^1 is H, a lower alkyl group or $N(R^3)_2$, R^2 is N, S or O, and R^3 is H or a lower alkyl group, and a pharmaceutically-acceptable oxidizing agent for leuco forms of the dye. The second solution is a rinse composition for removing unretained stain composition. The use which is taught for such kit includes the step of sequentially rinsing the oral cavity with that stain composition which is selectively retained by cancerous and precancerous oral tissues. The dye in the stain composition consists essentially of a dye which is either Azure B, Azure C or Brilliant Cresyl Blue. The oral cavity is then rinsed with a rinse composition for removing unretained stain composition.

(a) TITLE OF THE INVENTION

METHODS AND COMPOSITIONS FOR IN-VIVO DETECTION OF ORAL CANCERS AND PRECANCEROUS CONDITIONS

(b) TECHNICAL FIELD TO WHICH THE INVENTION RELATES

This invention relates to *in vivo* methods for detection of premalignant oral lesions and oral carcinomas. The invention also pertains to compositions for carrying out such diagnostic procedures.

(c) BACKGROUND ART

In vivo diagnostic procedures for detection of premalignant oral lesions or oral carcinomas, employing dye compositions which are selectively retained by tissues rendered abnormal due to dysplasia, hyperplasia, tumorigenesis, and other active surface lesions, are known in the art. For example, procedures employing fluorescein or fluorescein derivatives are disclosed in Chenz, Chinese Journal of Stomatology (27:44-47 (1992)) and Filiurin (Stomatologiia (Russian) 72:44-47 (1993)). These procedures involve application of the dye, followed by examination under ultraviolet light to detect the cancerous/precancerous tissue, which is selectively fluorescent.

Another prior art procedure involves *in vivo* application by rinsing with toluidine blue O, followed by normal visual examination to detect any selectively stained tissue. Such procedures are disclosed, for example in the U.S. Patent to Tucci, et al. No. 5,372,801 and in U.S. Patent 4,321,251 to Mashberg. Toluidine blue, has been used for decades as a histopathological stain for *in vitro* use. Through this use, it has become known as a metachromatic dye, staining nuclei rich in DNA and RNA a purple to pink colour. The inherent deep blue colour of toluidine blue is changed to purple or pink when the dye is bound to a nucleic acid or other acidic cellular macromolecule. Of course, this type of staining is dependent on the dye gaining access to internal subcellular structures, e.g., the nucleus. Such access is readily obtained only by "fixing" a tissue sample of formaldehyde or other reagent that disrupts the cellular membrane without destroying general cellular structure.

In contrast to the mechanism involved with *in vitro* use, the staining of oral tissue *in vivo* by toluidine blue O is due to its ability to penetrate the enlarged and more accessible interstitial spaces of dysplastic tissue, which retains the dye longer than areas

containing normal tissue with its tight intercellular junctions. Empirically, the observation is made that toluidine blue simply does not penetrate normal intercellular spaces efficiently than those of normal tissue, and becomes temporarily localized, it diffuses out with time and does not remain bound to the cells as it would if it actually "stained" acidic substructures.

The Mashberg procedure in U.S. Patent No. 4,321,251, involves the application of the toluidine blue O solution as a rinse of the entire oral cavity, with gargling, following by rinses with water and acidic acid to remove the dye which is not retained by the cancerous or precancerous tissue. The preliminary Mashberg diagnosis is then confirmed by direct application of the toluidine blue O composition to the suspect site 10-14 days later.

The Tucci et al., U.S. patent No. 5,372,801, patent discloses an improved toluidine blue O composition for use according to the general Mashberg procedure.

An *in vivo* procedure involving use of Lugol's solution (iodine) and toluidine blue O was proposed for detecting oesophageal cancer synchronous with upper aerodigestive tract cancers in Papazian (Gastroenterology Clinique et Biologique (9:16-22, 1985). Because of possible toxic properties of fluorescein, iodine and methylene blue, it is considered that they are not suitable for indiscriminate application by oral rinse and/or gargling procedures. Toluidine blue O is not toxic, but has been expensive to synthesize and purify in commercial quantities.

(d) DESCRIPTION OF THE INVENTION

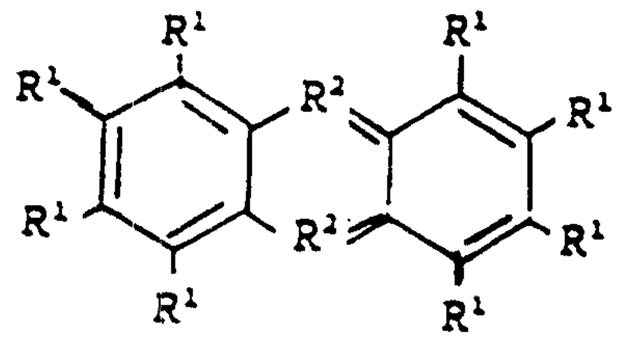
Accordingly, it would be advantageous, and it is a broad aspect of this invention, to employ dyes other than fluorescein, fluorescein derivatives, iodine, methylene blue or toluidine blue O in diagnostic procedures for the *in vivo* detection of premalignant oral lesions and oral carcinomas.

A object of another aspect of this invention is to provide *in vivo* diagnostic procedures and compositions useful therein which are especially useful in screening patients for possible oral cancer as part of routine dentist's or physician's examinations, or procedures e.g., periodic check-up's, dental cleaning, etc.

An object of yet another aspect of the invention is to provide such procedures and compositions which utilize dye stains which are more readily available and/or less expensive and less complicated to synthesize and/or purify then the dyes employed in prior art procedures.

An object of yet another aspect of the invention is to provide such *in vivo* procedures and compositions employing dyes which are less potentially toxic than prior art fluorescein derivatives and methylene blue and which may, therefore, be applied by rinsing the entire oral cavity and/or gargling.

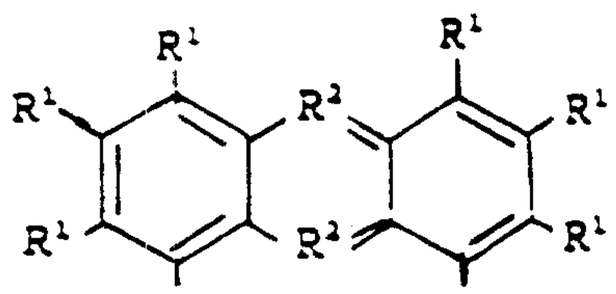
By one broad aspect, the present invention provides the use of a stain composition which consists essentially of a non-toxic dye other than toluidine blue O and methylene blue. The dye in the stain composition is selected from compounds having the structure



and ionic derivatives thereof, wherein R^1 is H, a lower alkyl group or $N(R^1)_2$, R^2 is N, S or O, and R^3 is H or a lower alkyl group. The use is for the *in vivo* detection of premalignant oral lesions and oral carcinomas. The use comprises the steps of sequentially first rinsing the oral cavity with the above stain composition which is selectively retained by cancerous and precancerous oral tissues, and then rinsing the oral cavity with a rinse composition for removing unretained stain composition. By variants of this use aspect of the invention, the dye in the stain composition is Azure B, Azure C or Brilliant Cresyl Blue.

By a second aspect of this invention, a kit is provided for the *in vivo* detection of cancerous and precancerous tissue. The kit includes a first solution comprising a biologic stain composition for rinsing the oral cavity, comprising a non-toxic dye other

than toluidine blue O and methylene blue, the dye in the biological stain composition having the structure



and ionic derivatives thereof, wherein R^1 is H, a lower alkyl group or $N(R^3)_2$, R^2 is N, S or O, and R^3 is H or a lower alkyl group, and a pharmaceutically-acceptable oxidizing agent for leuco forms of the dye. The kit includes a second solution comprising a rinse composition for removing unretained such stain composition.

By variants of the kit aspect of the invention, the dye in the stain composition is Azure B, Azure C or Brilliant Cresyl Blue.

By a second variant of the kit aspect of this invention, the pharmaceutically-acceptable oxidizing agent is hydrogen peroxide, urea peroxide, sodium perborate tetrahydrate, sodium percarbonate or calcium peroxide.

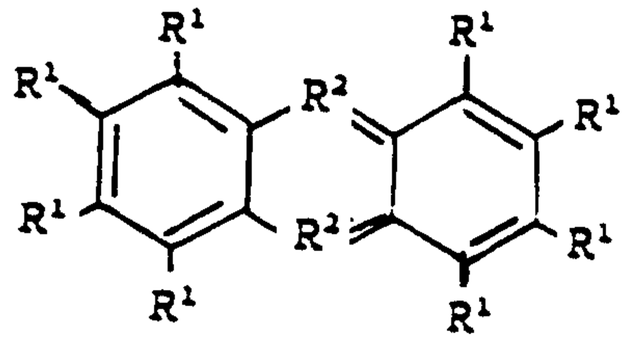
By a third variant of the kit aspect of the invention, and/or the above variants, the biological stain composition has a pH of 2.5 to 7.0. By one variation thereof, the biological stain composition has a pH of 4.0 to 5.0.

By a fourth variant of the kit aspect of the invention, and/or the above variants thereof, the provision of such pH is accomplished by means of a water-soluble buffer system which is acetic acid-sodium acetate, citric acid-sodium citrate, or citric acid-sodium phosphate.

By a fifth variant of the kit aspects of the invention, and/or the above variants thereof, the biological stain composition is in an aqueous solution including a pharmaceutically-acceptable, non-toxic, non-reactive alcohol. By one variation thereof, the alcohol comprises ethyl alcohol.

By a sixth variant of the kit aspect of the invention, and/or the above variants thereof, the concentration of the dye in the biological stain composition is 0.5% to 3.5% by weight. By one variation thereof, the concentration of the dye in the biological stain composition is 1% by weight.

Thus, by the present invention in its broad aspects, it has been found that non-toxic dyes having the structure



and ionic derivatives thereof, wherein R^1 is H, a lower alkyl group or $N(R^3)_2$, R^2 is N, S or O, and R^3 is H or a lower alkyl group, are effective for *in vivo* detection of premalignant oral lesions or oral carcinomas. The lower alkyl groups can be C_1-C_4, straight or branched chain groups, preferably methyl or ethyl groups. In general, these dyes are employed according to the same general procedure as disclosed by the Mashberg U.S. patent referred-to above, i.e., by sequentially rinsing the oral cavity with the dye stain composition, which is selectively retained by cancerous and precancerous tissues, and then rinsing the oral cavity to remove unretained stain composition.

The determination of whether a particular dye having the above-described structure is selectively retained can be readily ascertained by persons skilled in the art without undue experimentation, by using the dye in accordance with the general Mashberg procedure, described above. The potential toxicity of a particular dye can be readily determined by art-recognized techniques and standards, e.g., animal feeding studies.

Suitable compositions for the application of the dyes defined above are prepared by mixing the dye with a pharmaceutically-acceptable oxidizing agent which oxidizes any leuco form of the dye to the chromo form. The oxidizing agents are selected to be pharmaceutically acceptable, i.e., are non-toxic and do not cause undesirable side reactions which degrade the dye. Those skilled in the art will be able to identify suitable oxidizing agents for use in accordance with broad aspects of the invention by routine tests of known non-toxic mild oxidants. For example, in accordance with one presently preferred embodiment of the invention, it is preferred to use hydrogen peroxide, either directly or by reaction of known peroxide precursors with water in the compositions,

e.g., water soluble "per" compounds, e.g., urea peroxide, sodium perborate tetrahydrate, sodium percarbonate, calcium peroxide and the like.

The compositions of aspects of the invention are preferably formulated to yield a final solution which is substantially isotonic and has a pH in the range of 2.5 to 7.0, preferably 4.0 to 5.0. This is accomplished by adding an appropriate water-soluble buffer system. The presently preferred buffer system is acetic acid-sodium acetate. Other suitable buffer systems include citric acid-sodium citrate, or mixed acid-salt systems, e.g., citric acid-sodium phosphate and the like.

The solvent which is used to provide liquid dye compositions is an aqueous solvent. According to a preferred embodiment of the invention, the solvent includes a pharmaceutically-acceptable, i.e., non-toxic, non-reactive alcohol, e.g., ethyl alcohol. Such solvents do not appreciably interfere with the tissue staining mechanism and do not themselves contribute to the reduction of chromo forms of the dyes to the leuco forms.

Flavouring, stable to the oxidizing agent, may be added to improve the palatability of the dye solution if it is to be used as a "rinse".

The amount of the dye in the liquid compositions is preferably adjusted to yield a concentration of 1% by weight in the final composition, although higher concentrations can be employed and lower concentrations are at least partially effective, e.g., from 0.5 to 3.5 weight %.

Surprisingly, dyes having the above-defined structure, which are closely related to toluidine blue O, e.g., methylene blue and thionine, have proven to be ineffective, whereas other dyes closely related to toluidine blue O, e.g., Brilliant Cresyl Blue, Azure A, Azure B and Azure C are effective.

According to particular aspects of the present invention, Azure B and Azure C are employed in accordance with a presently preferred embodiment of the invention.

The invention in other broad aspects also contemplates compositions for use in accordance with the teachings of the invention and processes for manufacturing such composition, in which any leuco form of the dye which is present in the composition is oxidized to the chromo form, by inclusion of a pharmaceutically-acceptable oxidizing

agent in the composition, in the manner analogous to that disclosed in the Tucci et al., U.S.A. Patent 5,372,801.

(e) AT LEAST ONE MODE FOR CARRYING OUT THE INVENTION

EXAMPLES

The following examples are presented in order to illustrate practice of the invention to those skilled in the art.

EXAMPLE 1

Test compositions of Azure B, Azure C, Brilliant Cresyl Blue ("BCB") and thionine dyes and a control composition, toluidine blue O dye ("TBO") are prepared by mixing each of the dyes with the indicated ingredients in the following proportions (% by weight):

Ingredient	% by Weight
Purified Water, (U.S.P.)	83.86
Glacial Acetic Acid, (U.S.P.)	4.61
Sodium Acetate Trihydrate, (U.S.P.)	2.45
Ethyl Alcohol, (SD18)	7.48
Hydrogen Peroxide 30%, (U.S.P.)	0.41
IFF Raspberry IC563457 (colorant)	0.20
Dye	1.00

EXAMPLE 2

A rinse solution is prepared by mixing the following ingredients in the indicated proportions (weight %):

Purified Water, (U.S.P.)	98.70
Glacial Acetic Acid, (U.S.P.)	1.00
Sodium Benzoate, (U.S.P.)	0.10
IFF Raspberry IC563457 (colorant)	0.20

EXAMPLE 3

The clinical effectiveness of each of the test mixtures, compared to the TBO control mixture, is determined, utilizing the Mashberg protocol.

Patients are first screened for oral pathology employing the TBO control mixture. After identifying potential cancerous or precancerous pathology, all traces of the TBO are removed by repeated rinses with water and the acetic acid rinse solution. Those patients exhibiting oral pathology are then used as test subjects for each of the test mixtures. 2-3 cc of each test mixture is applied by painting the pathologic mucosal surfaces, followed by rinsing with the rinse mixture and water to remove excess dye composition. After examination with each dye and before testing with the next dye, all traces of the dye are removed from the lesion by repeated rinsing with the rinse mixture and water.

The Azure B test mixture provides equivalent staining intensity to TBO, localization of the lesions and delineation of the borders of the lesions. Adjacent unstained tissue shows slight dye accumulation, but not to the detriment of lesion discrimination.

The Azure C test mixture provides equivalent staining intensity to TBO, localization of the lesions and delineation of the borders of the lesions. Adjacent, uninvolved tissues are stained to a red/purple hue, but not to the detriment of lesion discrimination.

The Brilliant Cresyl Blue test mixture provides results similar to Azure B and Azure C.

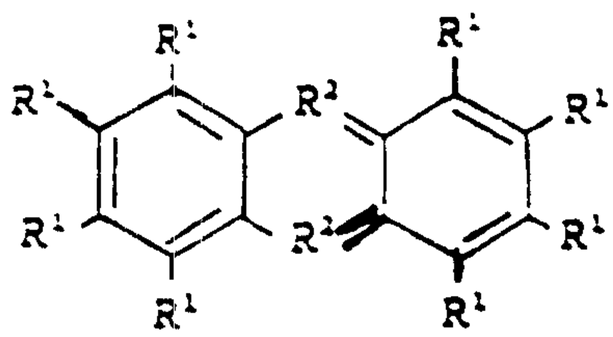
The Thionine test mixture provides a warm red background stain on both the stained and unstained tissues, such that the discrimination of the lesion borders is insufficient.

EXAMPLE 4

The procedures of Example 3 are repeated, except that the test and control compositions are applied to the oral mucosa by rinsing, with gargling, instead of by direct application to the locus of the suspect sites. Equivalent results are obtained.

CLAIMS

1. The use of a stain composition which consists essentially of a non-toxic dye other than toluidine blue O and methylene blue, said dye in said stain composition being selected from compounds having the structure



and ionic derivatives thereof, wherein R^1 is H, a lower alkyl group or $N(R^3)_2$, R^2 is N, S or O, and R^3 is H or a lower alkyl group, for the *in vivo* detection of premalignant oral lesions and oral carcinomas, comprising the steps of sequentially rinsing the oral cavity with a dye stain composition which is selectively retained by cancerous and precancerous oral tissues, and rinsing the oral cavity with a rinse composition for removing unretained stain composition.

2. The use as claimed in claim 1, wherein said dye in said stain composition consists essentially of Brilliant Cresyl Blue.

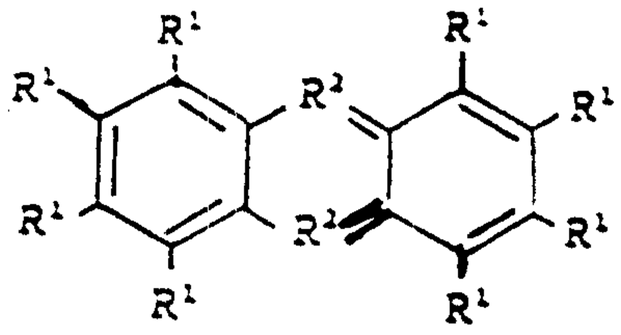
3. The use as claimed in claim 1, wherein said dye in said stain composition consists essentially of Azure B.

4. The use as claimed in claim 1, wherein said dye in said stain composition consists essentially of Azure C.

5. A kit for *in vivo* detection of cancerous and precancerous tissue, comprising:

(I) a first solution comprising:

(a) a biological stain composition for rinsing the oral cavity, comprising a non-toxic dye other than toluidine blue O and methylene blue, said dye in said biological stain composition having the structure



and ionic derivatives thereof, wherein R^1 is H, a lower alkyl group or $N(R^3)_2$, R^2 is N, S or O, and R^3 is H or a lower alkyl group, and

(b) a pharmaceutically-acceptable oxidizing agent for leuco forms of said dye; and

(II) a second solution comprising:

a rinse composition for removing unretained said stain composition.

6. The kit as claimed in claim 5, wherein said dye in said biological stain composition consists essentially of Brilliant Cresyl Blue.

7. The kit as claimed in claim 5, wherein said dye in said biological stain composition consists essentially of Azure B.

8. The kit as claimed in claim 5, wherein said dye in said biological stain composition consists essentially of Azure C.

9. The kit as claimed in any one of claims 5 to 8, wherein said pharmaceutically-acceptable oxidizing agent is selected from the group consisting of hydrogen peroxide,

urea peroxide, sodium perborate tetrahydrate, sodium percarbonate and calcium peroxide.

10. The kit as claimed in any one of claims 5 to 9, wherein said biological stain composition has a pH of 2.5 to 7.0.

11. The kit as claimed in claim 10, wherein said biological stain composition has a pH of 4.0 to 5.0.

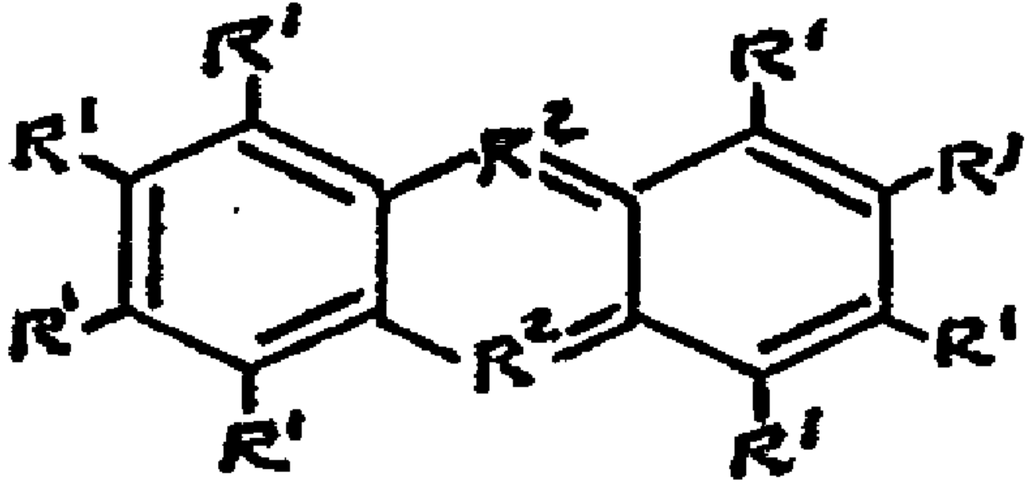
12. The kit as claimed in any one of claims 5 to 11, wherein said pH is accomplished by means of the use of a water-soluble buffer system, which is selected from the group consisting of acetic acid-sodium acetate, citric acid-sodium citrate, and citric acid-sodium phosphate.

13. The kit as claimed in any one of claims 5 to 12, wherein said biological stain composition is in an aqueous solution including a pharmaceutically-acceptable, non-toxic, non-reactive alcohol.

14. The kit as claimed in claim 13, wherein said alcohol comprises ethyl alcohol.

15. The kit as claimed in any one of claims 5 to 14, wherein the concentration of said dye in said biological stain composition is 0.5% to 3.5% by weight.

16. The kit as claimed in claim 15, wherein the concentration of said dye in said biological stain composition is 1% by weight.



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