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DRY ANALYTICAL ELEMENT FOR THE DETECTION OF
PROSTATIC ACID PHOSPHATASE

The present invention relates to clinical chemistry. In particular, it relates to an analytical
5 element and method for the determination of prostatic acid phosphatase in aqueous specimens, such as biological fluids.

Prostatic acid phosphatase (PAP) is also known as orthophosphoric monoester phosphohydrolase,
10 acid optimum, E.C. 3.1.3.2., and is a glycoprotein of 100,000 daltons molecular weight normally found in the prostate gland. It is a major constituent of semen. It is also present in serum at low levels, and is normally a mixture of enzymes contributed by various
15 tissues in the human body.

Assays for PAP began to be important several decades ago when it was discovered that serum acid phosphatase activity becomes elevated when metastatic cancer of the prostate or prostatomegaly is present.
20 Thus, a determination of the amount of PAP in body fluids can be useful for the diagnosis of such diseases and for the observation of their progress or remission. Various spectrophotometric assays have been developed using immunological and non-immunological techniques
25 and various enzyme substrates and dyes. Typically, PAP is evaluated from the amount or rate of formation of a chromophore (such as a dye). The chromophore is a coupled product of a phenol, resulting from the enzymatic hydrolysis of phospho monoester with, for
30 example, a diazonium dye in an acidic environment.

A representative early assay for PAP is described in US-A-3,002,893. Another described by Babson and others in Am.J.Clin.Pathol., 32, pages 88-91
35 (1959) suffers from a number of disadvantages such as the need for a blank to be run, lack of kinetic results and difficulty in formulating a dry assay.

Other assays using various phosphate substrates have been developed, but suffer from insensitivity, reagent instability or nonspecificity for the analyte. The most difficult problem is the instability of various reagents which, in a dry element, are stored for lengthy periods of time prior to their use. For example, sodium salts of the PAP substrate are unstable when stored for a period of time.

It would be highly desirable to have an assay for PAP which can be carried out in a dry analytical element which would provide a number of important advantages. To achieve this result, however, requires that a number of technical problems (noted above) be overcome.

The problems noted above with known assays have been overcome with an analytical element useful for the determination of prostatic acid phosphatase in an aqueous specimen comprising:

- a porous spreading zone containing
 - (a) a compound which reacts as a substrate with prostatic acid phosphatase to produce a phenol reaction product, and
 - (b) a diazonium or tetrazolium salt
- which is capable of reacting with the phenol reaction product to provide a chromophore,
- the element further comprising a buffer which maintains the element at a pH of from 3 to 6.5 when contacted with an aqueous specimen,
- the element characterized wherein the substrate compound is a nonhygroscopic aromatic phosphate.

This invention also provides a method for the determination of prostatic acid phosphatase comprising the steps of:



A) contacting an aqueous specimen suspected of containing prostatic acid phosphatase with the analytical element described above, and

5 B) detecting the formation of a chromophore as an indication of the presence of prostatic acid phosphatase in the specimen.

The present invention provides a dry analytical element for the effective and specific detection of prostatic acid phosphatase (PAP) in a
10 relatively short period of time at an acidic pH. The reagents in the element are highly stable so the element can be stored for a lengthy period of time before use without loss of activity and sensitivity. These advantages are achieved by using a relatively
15 nonhygroscopic aromatic phosphate substrate for PAP which does not absorb water to an appreciable extent. In a preferred embodiment, the diazonium or tetrazolium salt and buffer used in the assay are kept separated in the element prior to the assay.
20 This further improves storage stability.

The present invention relates to a determination (that is, detecting either the presence, amount or both) of PAP. In particular, the invention can be used to assay any aqueous fluid
25 suspected of containing PAP, and particularly biological fluids including, but not limited to, serum, urine, semen, lymph, plasma, whole blood and cerebral spinal fluid. It is also possible to assay fluid preparations of tissue such as preparations of the prostate gland. Preferably, human serum or
30 plasma is assayed with this invention.

In its broadest embodiment, the dry element of this invention has a porous spreading zone (or layer) which is fluid permeable and contains all of
35 the reagents needed for the detection of PAP. Optional materials can be in other optional (yet



preferred) zones. The elements are known as test strips, test slides or diagnostic devices.

The porous spreading zone is prepared from any of the known materials used for such zones as described, for example, in US-A-4,292,272, US-A-3,992,158, US-A-4,258,001, US-A-4,430,436 and related U.S. patents, and JP 57(1982)-101760. Preferred spreading zones are those described in US-A-3,992,158 as "blush polymer" zones.

10 Preferably, the elements have at least one other zone which can contain one or more reagents, needed for the assay. Such a zone is often known in the art as a reagent or registration zone, but it can also include a second porous spreading zone if
15 desired or printed layers located on other zones. The zones are generally in fluid contact with each other, meaning that fluids, reagents and reagent products can pass or be transported between superposed regions of adjacent zones, unless of
20 course, a reagent is immobilized in some manner so it will not migrate within or without a zone.

Preferably, the zones are separately coated as superposed layers on an inert support (see Example 1 below). The reagent zones or layers can be composed
25 of one or more binder materials [such as gelatin and other colloidal materials, hydrophilic polymers such as poly(vinyl alcohol), acrylamide polymers, vinylpyrrolidone polymers and others known in the art] in which reagents are incorporated.

30 The assay of this invention is carried out with the following sequence of reactions at a pH of from 3 to 6.5:

(a) PAP in a specimen is reacted with the nonhygroscopic aromatic phosphate substrate in the
35 element to provide a phenol reaction product, and

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(b) the phenol reaction product is coupled (or reacted) with a diazonium or tetrazolium salt to provide a detectable chromophore.

In an optimized assay, the conditions and amounts of reagents used in step (a) are adjusted so it is the rate limiting step of the assay.

The substrate for the analyte is relatively nonhygroscopic. The substrate is particularly less hygroscopic than substrate having sodium or potassium cations. The substrate is also at least partially water-soluble at the pH used in the assay.

The substrate also contains a phosphate group on an aromatic ring, and the phosphate group is cleaved by PAP to leave a hydroxy group on the ring of the resulting phenol reaction product. By phenol is meant any aryl hydroxide (substituted or unsubstituted) which is derived from any of the substrates described below. Preferably, the substrate is a nonhygroscopic salt of an aryl phosphate ester. Such aryl phosphate esters include, but are not limited to, *p*-nitrophenyl phosphate, phenolphthalein monophosphate, phenolphthalein diphosphate, thymolphthalein monophosphate, indoxyl phosphate, phenyl phosphate, α -naphthyl phosphate, β -naphthyl phosphate, β -glycerol phosphate acid; *o*-carboxyphenyl phosphate acid, *o*-methylfluorescein phosphate, 4-(4-nitro-2-methylsulfonylphenylazo)-naphthol-1-phosphate and others which would be readily apparent to one skilled in the art, including those described in US-A-4,983,512. Preferably, the aryl phosphate ester is a naphthyl phosphate ester, and α -naphthyl phosphate esters are most preferred.

Many of the aryl phosphate esters are commercially available, or they can be prepared using known reagents and procedures such as by reacting the appropriate phenol or naphthol with phosphoryl

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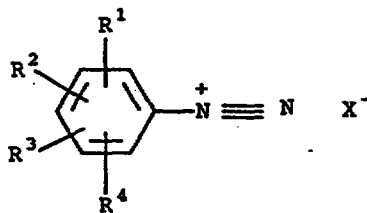


chloride, removing the halogen and isolating the resulting phosphate ester.

The aryl phosphate ester is used in the form of a relatively nonhygroscopic salt. Generally such a salt has one or more divalent cations, such as alkaline earth cations (for example, magnesium and calcium). Preferably, calcium salts are used with the hemicalcium salt being most preferred. It is believed that the noted cations stabilize the phosphate ester by being less hygroscopic than sodium ion, and because of its ability to absorb water molecules, thereby keeping the portion of the phosphate ester which reacts relatively free from contact with water. The substrate is thus less likely to be hydrolyzed.

The diazonium and tetrazolium salts useful in the practice of this invention must be those capable of reacting with a phenol reaction product generated by the action of PAP on the substrate, to produce a chromophore at a pH in the range of from 3 to 6.5. A diazonium salt is generally an organic salt of a compound having a diazonium radical. Aromatic diazonium salts are preferred.

Particularly useful diazonium salts include, but are not limited to, those having the following structure:



wherein R¹, R², R³ and R⁴ are independently hydrogen, halo (such as chloro, bromo or iodo), alkyl of 1 to 12 carbon atoms (such as methyl, ethyl, isopropyl, *t*-butyl, *sec*-butyl, *n*-butyl, pentyl, octyl, isononyl



and dodecyl), nitro, cyano, alkoxy of 1 to 6 carbon atoms (such as methoxy, ethoxy, pentoxy, isopropoxy, *t*-butoxy and hexoxy), aryloxy of 6 to 10 carbon atoms in the aromatic ring, including aryloxy substituted
5 with alkoxy and alkyl as defined above (such as phenoxy, naphthoxy, tolyloxy and *p*-nitrophenoxy), aryl of 6 to 10 carbon atoms (such as phenyl, naphthyl, *p*-methylphenyl, *m*-ethoxyphenyl, *p*-cyanophenyl and *o*-methoxyphenyl), acyl of 1 to 12
10 carbon atoms (such as acetyl, propionyl, benzoyl and butyryl), and substituted or unsubstituted carbamoyl or sulfamoyl (such as carbamoyl, sulfamoyl, *N,N*-dimethylcarbamoyl and *N,N*-diethylsulfamoyl). Preferably, R^1 , R^2 , R^3 and R^4 are independently
15 hydrogen, methyl, chloro, *N,N*-diethylsulfamoyl, nitro or methoxy.

In addition, any two of these radicals at adjacent positions on the ring can be taken together to represent an 6- to 10-membered fused aromatic ring
20 system, including both heterocyclic or carbocyclic fused aromatic rings. Such rings can include carbon, nitrogen, oxygen or sulfur atoms in the ring structure. Preferably, 6- to 10-membered carbocyclic ring systems are formed in this manner.

25 It is understood that R^1 , R^2 , R^3 and R^4 are not any groups that could interfere either with the diazotization reaction used in the assay to provide a diazonium salt, or the coupling reaction used in the assay to effect azo dye formation.

30 In the salt noted above, X^- represents any suitable anion such as halide (for example, chloride, bromide, fluoride and iodide), tetrafluoroborate, chlorozincate, hemizinc chloride, nitrate, perchlorate, *p*-toluenesulfonate and others readily
35 apparent to one skilled in the art.

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Representative useful diazonium salts include, but are not limited to, N',N'-diethyl-4-methoxymetanilamide diazonium salt (known as Fast Red-ITR salt), 4-chloro-2-methylbenzendiazonium salt (known as Fast Red TR salt), diazotized 2-methoxy-5-chloroaniline (known as Fast Red RC salt) and diazotized 5-nitro-2-amino-1-methoxybenzene (known as Fast Red B). The Fast Red TR salt is most preferred.

Tetrazolium salts are organic salts in which the organic portion contains one or two tetrazole rings, generally with aryl substituents, at various positions. Tetrazolium salts having two tetrazole rings can be coupled so as to provide a diphenyl group with the tetrazole rings in the two para positions.

Many useful diazonium and tetrazolium compounds are described for example, in US-A-3,905,872, US-A-4,772,553, US-A-4,892,817 and US-A-4,892,833 and Japanese Patent Publication 63/088,000.

The relatively nonhygroscopic aromatic phosphate substrate and diazonium or tetrazolium salt described above can be incorporated into the element of this invention in any suitable location. In the simplest embodiment, they are incorporated into the porous spreading zone (or layer) of a single-zoned element. In other embodiments containing a multiplicity of zones or layers, the noted reagents can be located together or separately in one or more of the zones. Preferably, they are in the same layer of a multilayer element.

The element also contains one or more buffers which maintains the element at a pH of from 3 to 6.5 during the assay. Useful buffers are well known in the art and include, for example, citrate, acetate, succinate, phthalate, glutaric and N-(2-acetamido)iminodiacetic, and the corresponding free

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acids. The buffers are preferably located in any of one or more zones of the element different from that zone containing the substrate. The element is preferably buffered to a pH of from 4.5 to 6.

5 It also is preferred that the element contain one or more accelerators which enhance the enzymatic reaction of the analyte and production of dye [see Saini and others, J. Biol. Chem., 256(20), pages 10453-10455, 1981 and Buchwald and others, 10 J. Biol. Chem., 259(4), pages 2208-2213, 1984]. Such compounds are generally straight or branched chain diols having from 3 to 8 carbon atoms. They include, but are not limited to, 1,4-butanediol, 1,5-pentanediol, 1,2-propanediol, glycerol, 2,3- 15 butanediol, 1,6-hexanediol, 1,5-hexanediol and others readily apparent to one skilled in the art. These accelerators are preferably in a zone other than the zone containing the substrate. Most preferably, the accelerators and buffers are in the same zone.

20 The amounts of reagents incorporated into the element of this invention are well within the skill of the ordinary worker in the art. More specifically, the aromatic phosphate substrate is generally present in an amount within the range of 25 from 0.005 to 5 g/m² (dry weight), with an amount of from 0.05 to 1 g/m² being preferred. The diazonium or tetrazolium salt is generally present in an amount within the range of from 0.005 to 5 g/m² (dry weight), with an amount of from 0.05 to 3 g/m² being 30 preferred. The accelerator, when used, is generally present in an amount within the range of from 0.05 to 10 g/m² (dry weight), with an amount of from 0.5 to 6 g/m² being preferred.

35 A variety of different elements, depending upon the method and equipment for assay, can be prepared in accordance with this invention. They can



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be configured in a variety of forms and shapes, including elongated tapes of any desired width, sheets, slides or chips.

In a preferred embodiment, a multilayer analytical element of this invention comprises a nonporous, transparent support having thereon, in order and in fluid contact:

a reagent layer containing a suitable buffer as noted above, and

10 a porous spreading layer containing the aromatic phosphate substrate and diazonium or tetrazolium salt as described above.

In an even more preferred embodiment, the reagent and porous spreading layers are separated by a hydrophilic subbing layer, such as a layer prepared from gelatin or other colloidal binder materials, vinyl pyrrolidone, acrylamide or N-alkyl-substituted acrylamide polymers (such as an N-isopropylacrylamide polymer), or other materials well known in the art.

20 The assay of this invention can be manual or automated. In general, the element is used by physically contacting it with the test specimen (for example from 1 to 200 μ l) suspected of containing prostatic acid phosphatase under ambient conditions (although other temperatures can be used). The specimen and reagents become mixed within the one or more zones of the element and react to provide a dye.

30 After specimen application, the element is exposed to any conditioning, such as incubation, heating or otherwise, that may be desirable to quicken or otherwise facilitate obtaining a test result.

Generally within 5 minutes, and preferably within 3 minutes, a first spectrophotometric measurement is made of any chromophore formed in the element. Since PAP is an enzyme which reacts over

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time, generally a second measurement is taken a few minutes later to provide a rate response.

Chromophore formation can be measured with suitable reflection or transmission spectrophotometric

5 equipment and procedures as a measure of analyte activity and presence in the test sample.

The chromophore is generally measured at a wavelength which is within the range of from 350 to 800 nm depending upon the reactants used, with
10 measurement at a wavelength of from 400 to 650 nm being preferred. Measuring at higher wavelengths,, such as from 550 to 650 nm (most particularly at 600 nm), avoids interference from bilirubin.

The following examples are included to
15 illustrate the practice of this invention, and are not meant to be limiting in any way. The materials used in the examples were obtained as follows:

ESTANETM 5715 polyurethane resin from B.F. Goodrich,

20 TRITONTM X-405 nonionic surfactant from Rohm and Haas,

ZONYL FSNTM nonionic surfactant from DuPont, the α -naphthyl phosphates from American BioOrganics or Sigma Chemical Co.,

25 Fast Red TR salt from Sigma Chemical Co., and the remainder of the materials from Eastman Kodak Company or other commercial sources, or they were prepared using standard procedures and readily available starting materials.

30 As used herein, one I.U. represents the International Unit for enzyme activity and is defined as the amount of enzyme activity required to catalyze the conversion of 1 μ mole of substrate to product per minute under standard pH and temperature conditions.

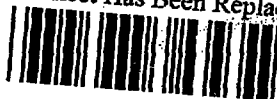
35 For PAP, the standard conditions are 37°C and a pH of 5.4.



Example 1 Multilayer Analytical Element for the
Determination of Prostatic Acid
Phosphatase and Comparative Elements

The element illustrated below was prepared by
5 formulating the materials of each layer into coating
dispersions using known procedures and solvents, and
coating them in the order shown using standard coating
equipment and procedures. The element of this
invention was compared in storage stability of the
10 reagents (particularly the nonhygroscopic aromatic
phosphate substrate) to the elements outside the
present invention using hygroscopic aromatic phosphate
substrates.

		Dry Layer Coverage (g/m ²)
Spreading Layer	Barium sulfate	107.6
	Cellulose acetate	8.6
	TRITON TM X-405 surfactant	2.1
	Fast Red TR salt	0.7
	α -Naphthyl phosphate, hemicalcium salt	0.4
	ESTANE TM polyurethane resin	1.4
Subbing Layer	Poly(N-isopropyl-acrylamide)	0.4
Reagent Layer	Gelatin	12
	Citrate buffer (pH 5.5)	5.5
	Bis(vinylsulfonylmethyl) ether hardener	0.2
	1,4-Butanediol	1.8
	1,5-Pentanediol	4.0
	Poly(ethylene-terephthalate) Support	



Comparative elements were similarly prepared having the following substrates present in the spreading layer at the same coverage (0.4 g/m²):

Comparative Element A: disodium salt of α -naphthyl phosphate,

Comparative Element B: monosodium salt of α -naphthyl phosphate, and

Comparative Element C: α -naphthyl phosphoric acid.

A sample (10 μ l) of a buffered solution (20 mmolar citrate, pH 5.1) containing PAP (47 I.U./liter) was applied to each element, and the rate of dye development was measured by reflectance spectrophotometry. Each of the elements was then kept at 21°C and 15% relative humidity for 1 week, and retested. The results of the two tests were compared to show the amount of loss of reagent stability as expressed in percent compared to an element used without room temperature storage.

20

Element	Rate Loss
Example 1	0.2%
Comparative Element A	6.6%
Comparative Element B	1.7%
Comparative Element C	1.5%

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Example 2 Determination of Prostatic Acid Phosphatase

The element of Example 1 was used to detect prostatic acid phosphatase at various concentrations, and after several days of storage.

In one set of experiments, individual elements were contacted with a sample containing 13 I.U./liter of the analyte and the resulting dye development rate was measured at 600 nm to determine

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rate response using a conventional reflectance spectrophotometer after the conditioning noted below.

Element	Rate Response	Percent of "Fresh Rate
Fresh*	0.01293	100
7 days @ 25°C	0.01269	98.1
14 days @ 25°C	0.01258	97.3
21 days @ 25°C	0.01248	96.5
28 days @ 25°C	0.01223	94.6

* Used immediately without storage

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These data indicate that the element of this invention is highly stable after storage at conditions typically found at a site of use.

Another set of experiments were carried out using a large number of elements like those of Example 1 to detect unknown amounts of prostatic acid phosphatase. The dye signal (D_R , reflectance density) formed in response to the analyte was measured and compared to the predicted value to determine precision of the assay. The results are shown in the following table.

TABLE

PAP (I.U./l)	Number of Tests	Rate (D_R /min)	Predicted PAP (I.U./l)	Coefficient of Variation
0.78	32	0.00082	0.74	6.5%
1.1	187	0.00139	1.1	3.5%
6.60	32	0.00743	6.58	2.4%
13.7	32	0.01294	13.7	1.4
20.0	32	0.01725	20.0	1.8%

20 Example 3 Preferred Element

A preferred element of this invention was prepared having the following layer arrangement and components:

		Dry Layer Coverage (g/m ²)
Spreading Layer	Barium sulfate	108.6
	Cellulose acetate	8.6
	TRITON™ X-405 surfactant	2.1
	Fast Red TR salt	0.7
	Hemicalcium α-naphthyl phosphate	0.2
	ESTANE™ polyurethane resin	1.4
Subbing Layer	Poly(N-iso-propylacrylamide)	0.39
Reagent Layer	Gelatin	14
	Sodium citrate buffer (pH 5.5)	4.8
	Citric acid	0.71
	Bis(vinylsulfon-ylmethyl)ether hardener	0.2
	1,4-Butanediol	1.8
	1,5-Pentanediol	4.0
	ZONYL™ FSN nonionic surfactant	0.2
	Poly(ethylene-terephthalate)	
	Support	

Example 4 Comparison of Elements with Diazonium Salt in Different Locations

5 This example compares the performance of an analytical element of this invention with one outside the scope of this invention whereby the diazonium salt is located in a layer other than the porous spreading layer.

10 The element of Example 1 was compared in the determination of PAP with the performance of a similar element whereby the Fast Red TR salt was located in the

reagent layer. The dye development was evaluated at several concentrations of PAP using the procedure and equipment described in Example 1. The results are presented in the following Table. The rate responses were measured immediately after manufacture and a second time (identified as "aged") after storage at 21°C and 15% relative humidity for four weeks.

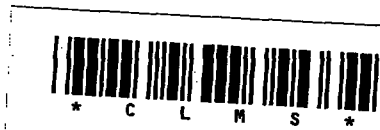
Concentration of PAP (I.U./liter)	Rate Response*			
	Reagent Layer		Spreading Layer	
	Initial	"Aged"	Initial	"Aged"
3	0.010	0.001	0.003	0.003
17	0.015	0.002	0.014	0.013
47	0.018	0.005	0.029	0.029

*Density change per minute

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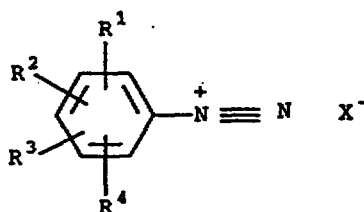
These results indicate that storage stability of the element was reduced when the diazonium salt was placed in the hydrophilic reagent layer containing the accelerators and buffer. Placement of the salt in the more hydrophobic spreading layer improves the storage stability of the element.

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CLAIMS

1. An analytical element useful for the determination of prostatic acid phosphatase in an aqueous specimen comprising:
 - 5 a porous spreading zone containing
 - (a) a compound which reacts as a substrate with prostatic acid phosphatase to produce a phenol reaction product, and
 - (b) a diazonium or tetrazolium salt
 - 10 which is capable of reacting with the phenol reaction product to provide a chromophore,the element further comprising a buffer which maintains the element at a pH of from 3 to 6.5 when contacted with an aqueous specimen,
 - 15 the element characterized wherein the substrate compound is a relatively nonhygroscopic aromatic phosphate.
2. The element as claimed in claim 1 wherein the nonhygroscopic aromatic phosphate substrate
 - 20 is an alkaline earth salt of an aryl phosphate ester.
3. The element as claimed in claim 1 wherein the substrate is the hemicalcium salt of α -naphthyl phosphate ester.
4. The element as claimed in any of claims
 - 25 1 to 3 wherein the diazonium salt has the structure:



- wherein R^1 , R^2 , R^3 and R^4 are independently hydrogen, halo, alkyl, nitro, cyano, alkoxy, aryloxy, aryl, acyl, carbamoyl or sulfamoyl, or any two of said radicals
- 30 which are at adjacent positions on the aromatic ring



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can be taken together to form an 6- to 10-membered fused aromatic ring, and X^- is an anion.

5 5. The element as claimed in any of claims 1 to 4 wherein the porous spreading zone is a blush polymer spreading layer coated on a nonporous, transparent support.

10 6. A multilayer analytical element for the determination of prostatic acid phosphatase comprising a nonporous, transparent support having thereon, in order and in fluid contact:

 a reagent layer containing a buffer which maintains the element at a pH of from 3 to 6.5 when contacted with an aqueous specimen, and

15 a porous spreading layer containing
 (a) a compound which reacts as a substrate with prostatic acid phosphatase to produce a phenol reaction product, and

 (b) a diazonium or tetrazolium salt which is capable of reacting with the phenol reaction product to provide a chromophore,

20 the element characterized wherein the substrate compound is a relatively nonhygroscopic aromatic phosphate.

25 7. The element as claimed in claim 6 further comprising an accelerator in the reagent layer.

 8. A method for the determination of prostatic acid phosphatase comprising the steps of:

 A) contacting an aqueous specimen suspected of containing prostatic acid phosphatase with an analytical element comprising:

30 a porous spreading zone containing
 (a) a relatively nonhygroscopic aromatic phosphate which reacts as a substrate with prostatic acid phosphatase to produce a phenol reaction product,
35 and

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(b) a diazonium or tetrazolium salt which is capable of reacting with the phenol reaction product to provide a chromophore,

the element further comprising a buffer which
5 maintains the element at a pH of from 3 to 6.5 during contact with the specimen, and

B) detecting the formation of the chromophore as an indication of the presence of prostatic acid phosphatase in the specimen.

10 9. The method as claimed in claim 8 wherein the aqueous specimen is human serum or plasma.

10. The method as claimed in either of claims 8 and 9 wherein the chromophore is detected at a wavelength in the range of from 550 to 650 nm.

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