Description

This invention relates to clinical chemistry. It provides an analytical element for the assay of salicylate.

The determination of salicylate in biological fluids such as human serum, has diagnostic significance. Acetylsalicylic acid (aspirin) is used as an analgesic and as an anti-inflammatory drug for arthritis. It rapidly hydrolyses to salicylate which has the therapeutic affect and a long half life. The therapeutic level as an analgesic is up to 20 mg/dl. For arthritis the level is up to 30 mg/dl. Problems such as headaches, tinnitus, flushing and hyperventilation occur at higher salicylate levels followed by imbalances in the acid-base level. Salicylate levels above 60 mg/dl can be lethal.

U.S. Patent 4,416,983 discloses a solution method for the determination of salicylate in body serums. The method is based on the following chemical reaction:

$$\text{salicylate} + \text{NAD(P)H} + O_2 \xrightarrow{\text{salicylate hydroxylase (EC 1.14.13.1)}} \text{pyrocatechol} + CO_2 + H_2O + NAD^+$$

$$\text{pyrocatechol} + \text{hydrazone} + O_2 \xrightarrow{\text{tyrosinase (EC 1.10.3.1) or (EC 1.14.18.1)}} \text{coloured material} + H_2O$$

The method as carried out in U.S. Patent 4,416,983 is a wet assay in which the two chemical reactions listed above are carried out simultaneously in one vessel. That is, all the chemical reagents needed to conduct the reactions are included in a single solution to which the salicylate contained in the sample is added.

That method as disclosed is not adaptable for dry analytical elements. The problem is that elements in which all of the reagents are included in a single layer have poor storage life. Work in our labs has shown that the life of the element as measured by its ability to respond to salicylate in a sample, is very short. The salicylate hydroxylase activity drops upon continued exposure to the hydrazone coupling agent.

The present invention provides multilayer analytical elements comprising a support coated in the following order, top down to the support:

a) a spreading layer,

b) a dye layer comprising tyrosinase and a hydrazone coupling agent,

c) a reagent layer comprising salicylate hydroxylase and nicotinamide adenine dinucleotide (NADH), wherein

d) the dye layer has a pH greater than 6.5;

e) the dye layer and reagent layer are separated by a barrier layer that prevents passage of molecules having a molecular weight in excess of 5,000, and

f) the hydrazone coupling agent is water insoluble at a pH above 6.5; or

a) a spreading layer;

b) a reagent layer comprising salicylate hydroxylase, NADH, tyrosinase and a hydrazone coupling agent, wherein

c) the hydrazone and the salicylate hydroxylase are in separate zones of the reagent layer separated by a barrier zone that prevents passage of molecules having a molecular weight greater than 5,000;

d) the zone in which the hydrazone coupling agent is located has a pH greater than 6.5 and;

e) the hydrazone is water insoluble at a pH above 6.5.

The present invention represents an unexpected improvement over the prior art. The colored hydrazone catechol complex is easily measured because of its high extinction coefficient. The complex is sufficiently formed within 5 minutes to allow effective measurement of the salicylate concentration colorimetrically. Due to the physical separation of the salicylate hydroxylase from the hydrazone coupling agent, the element stability during storage is greatly lengthened and response is increased. The use of a TiO_2 pigmented spreading layer also reduces many spectral and turbidity driven interferences seen in wet element systems.

In this element, the two reactions described above upon which the assay is based are carried out sequentially in separate layers or zones of the element. The barrier layer does not allow passage of

molecules having a molecular weight larger than 5,000. Salicylate hydroxylase has a reported molecular weight of 91,000. Thus it is trapped in the bottom layer below the barrier layer. The hydrazone coupling agent as a hydrochloride is water soluble, but at pH above 6.5, the hydrochloride is removed and the dye precipitates out of solution. In a preferred analytical element of this invention, the hydrazone coupling agent is coated in an unbuffered gelatin layer at pH 5.5 over a pH 8 buffered barrier layer. Once coated and dried, the top barrier layer is also at a pH of 8, from buffer that has diffused up from the lower gelatin layer. As the pH increases, the 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) drops out of solution and becomes crystalline in nature. In this form the hydrazone coupling agent cannot diffuse down through the intervening barrier layer that separates it from salicylate hydroxylase. Thus, the hydrazone coupling agent differs from that disclosed in U.S. Patent 4,416,983 in that the hydrazone coupling agent disclosed in that is water-soluble at a pH above 6.5.

The multilayer elements of the present invention can be used in a method for assaying biological fluids for salicylate comprising the steps of:

a) spotting the slide analytical element described above with a sample suspected of containing salicylate;

b) allowing an incubation time of at least about 3 to 5 minutes; and

c) making a colorimetric determination of the colorimetric qualitative assay of the salicylate present in the sample.

The element of this invention can be used to assay salicylate qualitatively and quantitatively in biological fluids in animals or humans, but preferably of humans. Such fluids include, but are not limited to, whole blood, plasma, sera, lymph, bile, urine, spinal fluid, sputum, perspiration and the like as well as stool secretions. It is also possible to assay fluid preparations of human or animal tissue such as skeletal muscle, heart, kidney, lungs, brains, bone marrow, skin and the like.

Elements of the invention can be configured in a variety of forms, including elongated tapes of any desired width, sheets, slides or chips.

The elements can be used in manual or automated assay techniques. In general, in using the elements, salicylate determination is made by taking the element from a supply roll, chip packet or other source and physically contacting it with a sample (for example, up to 200 µl) of the liquid to be tested so that the sample and reagents interact sequentially within the element become mixed. Such contact can be accomplished in any suitable manner, for example, by dipping or immersing the element into the sample or, preferably, by spotting the element by hand or machine with a drop of the sample with a suitable dispensing means.

After sample application, the element is incubated, for a period of up to 5 minutes, to facilitate color development. By incubation, we simply mean that the reagents are maintained in contact with each other for a period of up to 5 minutes before color measurements are made.

The dry analytical elements of this invention are multilayered. At least one of the layers is preferably a porous spreading zone. The other layers may include a reagent layer and a dye layer. The reagent layer may include a barrier zone and a reagent zone. All of the foregoing layers are coated on a support. The layers are generally in fluid contact with each other, meaning that fluids, reagents and reaction products (for example, color dyes) can pass or be transported between superposed regions of adjacent zones except for the restricted passage of molecules through the barrier layer or zone. In other words, when the element is contacted with an aqueous fluid, all reagents of the analytical composition of this invention mixed sequentially as stated hereinbefore and can readily move within the element as a composition. Each layer can be separate or two or more zones can be separate areas in a single layer of the element. Besides the references noted above, suitable element components are described also, for example, in U.S. Patents 4,042,335; 4,132,528 and 4,144,306.

Useful spreading layers can be prepared using fibrous materials, either mixed with a suitable binder material or woven into a fabric, as described in U.S. Patent 4,292,272, polymeric compositions or particulate materials, for example a blush polymer such as disclosed in U.S. Patent 3,992,158, beads bound together with or without binding adhesives, as described in U. S. Patents 4,258,001 and 4,430,436 and Japanese Patent Publication 57(1982)-101760. Particularly useful spreading layers comprise barium sulphate or titanium dioxide. Since the sample is generally applied directly to the spreading layer, it is desirable that the spreading layer be isotropically porous, meaning that the porosity is the same in each direction in the layer as caused by interconnected spaces or pores between particles, fibers or polymeric strands.

The layers can be coated on transparent supports such as polyethylene terephthalate. Other supports are well known in the art.

The elements of this invention can also contain one or more other addenda commonly put in the elements for various manufacturing or operational advantages. Such addenda include surfactants, buffers, solvents, hardeners and other materials known in the art.

The following examples clearly establish the improved aspects of the present invention.

Comparative Example: A Reagent Element in which all of the Reagents are included in a single layer

When all the reaction chemicals are contained in a single element layer the life of the element, as measured by its ability to respond to salicylate, was very short. The salicylate hydroxylase activity dropped upon continuous exposure to the hydrazone (MBTH). Although the reaction ingredients could function together in a wet system, separation was required to achieve a practical element life in a dry system.

Combined System Element:		g/m^2
Spreading Layer	$BaSO_4$ pigment	107.6
	Estane® 5715 binder	1.08
	Cellulose acetate binder	8.6
	(poly-N-isopropylacrylamide) binder	.4
	Triton® X-405 surfactant	2.1
Reagent Layer	Deionized gelatin binder	12.0
	Potassium phosphate buffer	3.0
	Sodium chloride	1.5
	Bis(vinylsulfonylmethyl) ether hardener	.24
	3-Methyl-2-benzothiazolinone hydrazone hydrochlorine (MBTH)	.25
	Tyrosinase	100,000 units/m^2
	Nicotinamide adenine dinucleotide (NADH)	.76
	Salicylate hydroxylase	1,000 units/m^2
	pH 7.6	
Support	Poly(ethylene terephthalate)	

Estane 5715 binder is a commercially available polyurethane binder.

When spotted with serum containing 100 mg/dL of salicylate the following reflection density response was measured over time at 540 nm.

Initial Time	Two Days at 25 °C	Five Days at 25 °C
.64	.37	.21

Addition of more salicylate hydroxylase to the element would allow it to temporarily regain its initial response to salicylate. Solution studies showed that it was the presence of MBTH that caused the loss of salicylate hydroxylase activity:

	Time Zero	30 Minutes at 4 °C
Salicylate Hydroxylase	33.4 u/L	36.2 u/L
Salicylate Hydroxylase with 3-Methyl-2-benzothiazolinone hydrazone hydrochlorine (MBTH)	33.4 u/L	28.2 u/L

Separation of the salicylate hydroxylase and the hydrazone was required to achieve adequate element storage life.

Example 1: The Present Invention

Element Structure and Content:	g/m²	Range g/m²
Spreading Layer		
TiO_2 pigment	50.0	20-100
Estane® 5715	2.5	.2-10
Cellulose acetate binder	6.9	2-20
Poly-N-isopropylacrylamide binder	.4	.05-2
Triton® X-405 surfactant	1.6	0-4
Oleyl PEG surfactant	.8	0-3
Reagent Layer		
Reagent zone		
Deionized gelatin binder	6.0	.2-15
Sodium chloride	.5	0-4
Bis(vinylsulfonylmethyl)ether hardener	.1	.01-1
3-Methyl-2-benzothiazolinone hydrazone	.4	.05-2
Tyrosinase	80,000 U/m²	5-300 K
pH	5.2	3-6.5
Barrier Zone		
Deionized gelatin binder	6.0	.2-15
Bicine buffer	1.5	.2-5
Sodium chloride	.5	0-4
Bis(vinylsulfonylmethyl)ether	.1	.02-1
Nicotinamide adenine dinucleotide	1.0	.1-4
pH	7.8	6.5-9.0
Reagent Zone		
Deionized gelatin binder	6.0	.2-15
Bicine buffer	1.5	.2-5
Sodium chloride	.5	0-4
Bis(vinylsulfonylmethyl)ether	.1	.02-1
Salicylate hydroxylase	576 U/m²	100-5 K
pH	7.8	6.5-9.0
Support		
Poly(ethylene terephthalate)		

When spotted with serum containing 100 mg/dL of salicylate the following density response was measured over time at 540 nm.

Initial Time	7 Days at 25 °C	14 Days at 25 °C	28 Days at 25 °C
1.27	1.27	1.27	1.25

The element was stable over time. The absolute response was greater than even the initial response in the case where the hydrazone and the salicylate hydrolase are in the same layer. The tyrosinase was moved into the same layer or zone with MBTH as neither could move in the element and they need to be in contact to function optimally. Separation is achieved even after rewetting with the sample as hardened gelatin will not allow passage of proteins or enzymes of greater than 5,000 molecular weight and the MBTH is not water soluble at a pH greater than 6.5. The combination of these two affects prevents the MBTH and the salicylate hydroxylase from coming into contact during manufacture or storage. Contact upon rewetting is not necessary as long as the catechol can readily diffuse between layers.

The MBTH is coated in a solution at pH 5.5 at which it is water soluble. Once it has been coated over the underlying gelatin layers containing buffer at pH 7.8 and the pH of the MBTH layer also increases to 7.8 and the MBTH becomes insoluble in water and thus immobile.

The usefulness of this element as a method of quantifying salicylate concentration in serum or other fluids is shown in the following table:

Reference Salicylate mg/dL	No. of Tests	Dr	Predicted Values	Within Run CV	Total CV
3.0	80	.13969	3.0	0.37%	0.51%
16.5	78	.72842	16.45	1.24%	1.30%
20.9	80	.79173	20.86	0.65%	1.47%
38.4	79	.97579	37.86	0.65%	0.88%
48.5	77	1.02077	48.31	0.73%	1.06%

The element gives accurate and precise results.

Claims

1. A multilayer analytical element comprising a support coated in the following order, top down to the support:
   a) a spreading layer,
   b) a dye layer comprising tyrosinase and a hydrazone coupling agent,
   c) a reagent layer comprising salicylate hydroxylase and nicotinamide adenine dinucleotide (NADH), wherein
   d) the dye layer has a pH greater than 6.5;
   e) the dye layer and reagent layer are separated by a barrier layer that prevents passage of molecules having a molecular weight in excess of 5,000, and
   f) the hydrazone coupling agent is water insoluble at a pH above 6.5.

2. The element of claim 1 wherein the barrier layer is a hardened gelatin.

3. The element of claims 1 or 2, wherein the dye layer, the barrier layer and the reagent layer each comprise hardened gelatin.

4. The elements of claims 1, 2 or 3, wherein the spreading layer comprises barium sulfate.

5. The element of claims 2 or 3, wherein the spreading layer comprises titanium dioxide.

6. The element of claims 1 2 or 3, wherein the hydrazone coupling agent is 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH).

7. The element of claim 6, wherein the pH of the barrier layer and the reagent layer is about 8.0.

8. A dry analytical element for assaying salicylate comprising a support having the following layers, top down to the support:
   a) a spreading layer;
   b) a reagent layer comprising salicylate hydroxylase, NADH, tyrosinase and a hydrazone coupling agent, wherein
   c) the hydrazone and the salicylate hydroxylase are in separate zones of the reagent layer separated by a barrier zone that prevents passage of molecules having a molecular weight greater than 5,000;
   d) the zone in which the hydrazone coupling agent is located has a pH greater than 6.5 and;
   e) the hydrazone is water insoluble at a pH above 6.5.

Patentansprüche

1. Mehrschichtiges analytisches Element, umfassend einen Träger, der von oben nach unten zum Träger hin in der folgenden Reihenfolge beschichtet ist:
   a) eine Ausbreitschicht,

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   a) eine Ausbreitschicht,

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b) eine Farbstoffschicht, die Tyrosinase und einen Hydrazonkuppler umfaßt,
c) eine Reagenzschicht, die Salicylathydroxylase und Nicotinamidadenindinucleotid (NADH) umfaßt, wobei
d) die Farbstoffschicht einen pH von größer als 6,5 aufweist;
e) die Farbstoffschicht und die Reagenzschicht durch eine Sperrschicht getrennt sind, die den Durchtritt von Molekülen, die ein Molekulargewicht von über 5 000 aufweisen, verhindert und
f) der Hydrazonkuppler bei einem pH von über 6,5 unlöslich ist.

2. Element nach Anspruch 1, bei dem die Sperrschicht eine gehärtete Gelatine ist.

3. Element nach Ansprüchen 1 oder 2, bei dem die Farbstoffschicht, die Sperrschicht und die Reagenzschicht jeweils gehärtete Gelatine umfassen.

4. Elemente nach Ansprüchen 1, 2 oder 3, bei denen die Ausbreitschicht Bariumsulfat umfaßt.

5. Element nach Ansprüchen 2 oder 3, bei dem die Ausbreitschicht Titandioxid umfaßt.

6. Element nach Ansprüchen 1, 2 oder 3, bei dem der Hydrazonkuppler 3-Methyl-2-benzothiazolinonhydrazonhydrochlorid (MBTH) ist.

7. Element nach Anspruch 6, bei dem der pH der Sperrschicht und der Reagenzschicht etwa 8,0 beträgt.

8. Trockenes analytisches Element zum Testen von Salicylat, das von oben nach unten zu dem Träger hin einen Träger mit den folgenden Schichten umfaßt:
a) eine Ausbreitschicht;
b) eine Reagenzschicht, die Salicylathydroxylase, NADH, Tyrosinase und einen Hydrazonkuppler umfaßt, wobei
c) das Hydrazon und die Salicylathydroxylase in getrennten Zonen der Reagenzschicht vorliegen, getrennt durch eine Sperrzone, die den Durchtritt von Molekülen, die ein Molekulargewicht von größer als 5000 aufweisen, verhindert;
d) die Zone, in der sich der Hydrazonkuppler befindet, einen pH von größer als 6,5 aufweist; und
e) das Hydrazon bei einem pH von über 6,5 wasserunlöslich ist.

Revendications

1. Un élément analytique multicouche comportant un support revêtu dans l'ordre suivant, du sommet vers le bas du support:
a) une couche de revêtement,
b) une couche de pigment comportant de la tyrosinase et un agent de couplage de type hydrazone,
c) une couche de réactif comportant de la salicylate hydroxylase et du dinucléotide d'adénine nicotinamide (NADH),
dans lesquelles
d) la couche de pigment présente un pH supérieur à 6,5;
e) la couche de pigment et la couche de réactif sont séparées par une couche barrière qui évite le passage des molécules présentant un poids moléculaire supérieur à 5 000, et
f) l'agent de couplage de type hydrazone est insoluble dans l'eau pour un pH supérieur à 6,5.

2. L'élément de la revendication 1, dans lequel la couche barrière est une gélatine durcie.

3. L'élément des revendications 1 ou 2, dans lequel la couche de pigment, la couche barrière et la couche de réactif comporte chacune de la gélatine durcie.

4. Les éléments de la revendication 1, 2 ou 3, dans lesquels la couche de revêtement comporte du sulfate de baryum.

5. Les éléments de la revendication 2 ou 3, dans lesquels la couche de revêtement comporte du dioxyde de titane.

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6. L'élément des revendications 1, 2 ou 3, dans lequel l'agent de couplage de type hydrazone est le chlorhydrate de 3-méthyl-2-benzothiazolinone hydrazone (MBTH).

7. L'élément de la revendication 6, dans lequel le pH de la couche barrière et de la couche de réactif est d'environ 8,0.

8. Un élément analytique sec pour un essai au salicylate, comportant un support ayant les couches suivantes, du sommet vers le bas sur le support:
   a) une couche de revêtement;
   b) une couche de réactif comportant de la salicylate hydroxylase, du NADH, de la tyrosinase et un agent de couplage de type hydrazone, dans lequel
   c) l'hydrazone et la salicylate hydroxylase sont dans des zones distinctes de la couche de réactif séparées par une zone de barrière qui évite le passage de molécules présentant un poids moléculaire supérieur à 5 000;
   d) la zone dans laquelle l'agent de couplage de type hydrazone est situé présente un pH supérieur à 6,5; et
   e) l'hydrazone est insoluble dans de l'eau pour un pH supérieur à 6,5.

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