(54) Title: ENZYMATIC DETERMINATION OF THEOPHYLLINE

(57) Abstract

The present invention provides a new methodology and test composition for determining the presence of theophylline in test samples. The methodology employs enzymes that utilize or recognize theophylline as a substrate to measure the concentration thereof in samples, including body fluids. This new approach utilizes enzymes as opposed to traditional methods which use antibodies for the recognition of theophylline. The enzymatic approach to theophylline determination is quick, simple and convenient and allows test systems to be made in liquid as well as in dry-chemistry formats. Various protocols, systems or methodologies may be used for assaying and relating the results to the amount of theophylline present.
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ENZYMATIC DETERMINATION OF THEOPHYLLINE

BACKGROUND OF THE INVENTION

Theophylline is a bronchodilator and respiratory stimulant used in the treatment of patients with asthmatic and allergic conditions. It is also used in the treatment of congestive heart failure and acute pulmonary edema. Benefits, as well as risks, from using this drug directly relate to its serum concentration. In order for the drug to be effective, a concentration of theophylline of about 10-20 mg/L level needs to be maintained in the blood. Theophylline levels of less than 10 mg/L are therapeutically ineffective and levels of more than 20 mg/L may be toxic to the patient. This toxicity may result in brain damage and death. Because the therapeutic advantage of the drug lies only within a narrow range of concentrations and because there is a large interpatient difference in drug elimination due to physiological differences, as well as diet or other prescribed drugs, it is important to monitor patients using this drug.

Theophylline has been measured by gas chromatography by Shah, J Pharm Sci 63(8), 1283 (1974) and by a combination of gas chromatography and mass-selective detector by Desage, et al., J Chromat 336(2), 285 (1984). It has been measured
the determination of theophylline. However, because these methods need cumbersome extractions, most clinical approaches today for the determination of theophylline use immunological methods which depend on antibodies for recognizing theophylline in the sample being tested. Such systems involve competitive protein binding where the antibody is the specific binding protein. After the reaction with antibodies takes place, the determination of theophylline varies depending on the particular assay, that is, the assay readout may be turbidimetric, nephelometric, radioactive or colorimetric depending on whether turbidity, radioactivity or color is produced. Examples of these systems are reported by Painter, et al., J Clin Lab Autom 3(3), 179 (1983), Samoszuk, et al., Therp Drug Mon 5(1), 113 (1983), Opheim, et al., Clin Chem 30(11), 1870 (1984), Boeckx and Munson, Therp Drug Mon 7(1), 95 (1985), Cook, et al., Res Comm in Chem Path & Pharm 13(3), 497 (1976), and Landesman, et al., Clin Chem 29, 1238 (1983).

Immunological systems have also been reported by Li et al., Clin Chem 27(1), 22 (1981), Davis and Marks, Ther Drug Mon 5(4), 479 (1983), Chang, et al., Clin Chem 28(2), 361 (1982), Hinds, et al., Clin Chem 30(7), 1174 (1984), Jolley,

In addition, enzymes have been used in an enzyme amplification assay, U. S. Patent No. 3,817,837. In this disclosure, enzymes are chemically bound to ligands and these enzyme-bound-ligand combine with receptors. The ligand may be a drug. The specific reaction of the ligand with the receptors gives the specificity to the reaction while the enzyme activity is utilized as a marker for the reaction. Therefore, the enzymes used have no enzymatic recognition of the drug. The use of these approaches are totally different to the presently described methodology which uses enzymes instead of antibodies or ligands for the recognition of theophylline.

It has been also reported in European Patent application number EP86300226.7, Jan 15, 1986, as well as in Clin Chem 25, 1370 (1979) that the activity of alkaline phosphatase, which acts by cleaving phosphate groups from a substrate, can be inhibited by theophylline. In this approach, the enzymatic reaction of alkaline phosphatase continues to be that of cleaving the phosphate bonds but this action is interfered with by the presence of theophylline. Again, in this disclosure, the theophylline test produced is one in which theophylline is not enzymatically utilized or changed
by the enzymatic reaction. By contrast, the present invention teaches that test systems can be produced, using enzymes which utilize or recognize theophylline and use it as a substrate for the quantitation of theophylline in fluids.

No theophylline utilizing enzyme has previously been available or described in the prior art. Moreover, since theophylline is a xanthine derivative, commercially available xanthine oxidases and xanthine dehydrogenases and related enzymes were tried for their ability to utilize theophylline. These attempts were unsuccessful. Although in humans theophylline is known to be metabolized primarily to 1,3 dimethyl uric acid and also to 1 methyl uric acid and 3 methyl xanthine (Cornish, H.H. and Christman, A.A., J Biol Chem 228, 315 (1957), no theophylline utilizing enzymes have been isolated or shown. However, recently three theophylline utilizing or recognizing enzymes have become available from GDS Technology Inc., P.O. Box 473, Elkhart, Indiana 46515. These enzymes were identified as 1. Enzyme T-090, 2. Enzyme T-060 and 3. Enzyme T-040.

These enzymes were used herein for the first time to develop and produce an enzymatic test for the determination of theophylline in samples such as body fluids, food extracts and medicinal compounds and compositions. The
tests that resulted from this enzymatic approach are rapid and convenient to perform. The advantages of the enzymatic approaches are 1) unitized reagent or test composition capability, 2) one step addition of sample to reagent or test composition, 3) a liquid system can be made to perform with instrument readout devices, and 4) the reagent or test composition can easily be incorporated into a solid-phase matrix.

BRIEF DESCRIPTION OF THE DRAWING

The figure shown in the drawing depicts absorbance curves for theophylline utilizing enzyme before and after contact with theophylline as further described in Example 3.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention basically consists of a system for the determination of theophylline by means of a theophylline utilizing or recognizing enzyme (or substance containing such enzyme) and optionally including the use of electron carriers. The determination is accomplished by measurement of a signal produced by the reaction of the enzyme with any theophylline present in the sample and converting or correlating the amount of signal generated to the amount of theophylline present in the sample. As used
herein, the term theophylline utilizing enzyme means an enzyme or substance containing an enzyme which either recognizes or utilizes theophylline to produce a signal which by itself or in conjunction with other reagents or means can be measured using visual, instrumental or other state of the art methodologies.

The principle of the test method is based on the utilization of theophylline by an enzyme. That is, the enzyme recognizes theophylline as a substrate and changes it to a different compound or product. Schematically, the system can be described as follows:

```
   enzyme
theophylline  -->  Product
```

As noted above, the methods disclosed and claimed herein involve detecting any signal produced by contact of theophylline with the theophylline utilizing enzyme which indicates that a specific enzymatic reaction has taken place. The signals produced are measured in a variety of ways as described herein. This process occurs in the presence or absence of added electron carriers, either electron acceptors or electron donors. Examples of electron acceptors are oxygen, nicotinamide adenine dinucleotide (NAD), dichlorophenolindophenol (DCPIP), phenazine methosulfate (PMS), methylene blue, cytochromes,
ferricyanide, etc. Examples of electron donors are reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced flavin adenine dinucleotide (FADH), etc.

The process of the present invention is otherwise carried out in the usual manner for enzymatic determinations, including optimized pH and temperature ranges in which theophylline utilizing enzymes are active. Moreover, such determinations are preferably carried out in a buffered environment.

In principle, all variants of the theophylline utilizing enzyme which can be used for the enzymatic determination of theophylline fall within the scope of the present invention.

The following represents various test systems wherein theophylline in a test sample can be determined using the methodology disclosed in the present specification:

1) Measuring the decrease of theophylline concentration in the system. In this system, the disappearance of the substrate theophylline can be measured by determining the decrease of absorbance at a wavelength of 272 nm, where theophylline has maximum absorbance. This is shown in Example 1 by using the theophylline enzyme T-060
and in Example 2 by using the theophylline enzyme T-090. This change can also be detected by measuring the reflectance, which is the inverse of the absorbance, or

2) Measuring a change of the oxidation-reduction potential of the system in either of two ways, a) measuring a change in the enzyme or enzyme complex system itself as shown in Example 3, wherein the enzyme characteristics change as can be seen by the changes that occur at wavelength 410 nm and 550 nm or measuring changes electrochemically, or b) by a change of an electron carrier added to the system such as, for example, the reduction of ferricyanide to ferrocyanide. Example 4 shows the absorbance change at 410 nm that occurs when ferricyanide changes to ferrocyanide in the presence of the theophylline enzyme T-090 as the reaction takes place. This change can also be measured by spectrophotometric or electrochemical methods, or

3) Measuring the appearance of any product associated with the enzymatic reaction of theophylline. The products produced in this reaction vary with the particular enzyme involved. For example, a theophylline enzyme can react by oxidizing, dehydrogenating or demethylating theophylline. Example 5 shows the appearance and measurement of formaldehyde when the theophylline enzyme T-040 was used. Example 6 shows the appearance and measurement of hydrogen peroxide when the theophylline enzyme T-060 was used.
The product formation can be further illustrated by the following reactions or processes:

5  i) When the enzyme is capable of oxidizing theophylline and converts theophylline (1,3 dimethyl xanthine) to 1,3 dimethyl uric acid utilizing oxygen as an electron acceptor. This is diagrammatically shown as follows:

\[
\text{enzymee} \\
\text{theophylline} + O_2 + H_2O \rightarrow 1,3 \text{ dimethyl uric acid} \\
\text{+ hydrogen peroxide}
\]

15  In this system, the hydrogen peroxide thus produced can be determined titrimetrically, potentiometrically, polarographically, colorimetrically as well as enzymatically. The enzymatic methods of measuring hydrogen peroxide are preferred since they are not only specific and reliable, but can also be combined in a simple way with the hydrogen peroxide of the above reaction to produce color. For example, a peroxidase method is described in Anal Biochem 105, 389, (1980). Using the theophylline enzyme T-60, Example 6 demonstrates that the hydrogen peroxide formation is proportional to the concentration of theophylline in the sample. Alternatively, the rate of oxygen consumption in accordance with the above general
equation can be measured, for instance, by gas chromatography and depolarization methods. The depolarization method utilizing oxygen electrodes (available from Yellow Spring Instruments, Yellow Spring, OH) is well known and also described in US Patent No. 3,838,011 and in J Appl Physiol 18, 1247 (1963).

ii) When the enzyme uses an acceptor other than oxygen such as ferricyanide, NAD, cytochromes, etc. to oxidize theophylline and produces 1,3 dimethyl uric acid and a reduced acceptor in the following manner:

\[
\text{enzyme} \\
\text{theophylline + oxidized acceptor} \rightarrow \text{1,3 dimethyl uric acid + reduced acceptor}
\]

In such a system, the product 1,3 dimethyl uric acid can be measured or determined by several methods. Example 7 describes one in which the absorbance at 292 nm is determined using the theophylline enzyme T-090. At such a wavelength 1,3 dimethyl uric acid absorbs optimally. Again, the increase in absorbance was found to be proportionate to the theophylline concentration in the sample.

Moreover, the concentration of theophylline in the sample can be determined using this reaction scheme by measuring the oxidation/reduction state of the electron
acceptors used. Examples 4 and 8 show a test method where ferricyanide is used as an acceptor and is reduced to ferrocyanide by the theophylline enzyme T-090. In Example 4 the decrease of ferricyanide is measured by measuring the decrease in absorbance at 410 nm wavelength as ferricyanide maximally absorbs at 410 nm and ferrocyanide has no absorption at that wavelength. It was again found that the decrease in absorbance was proportionate to the concentration of theophylline in the sample. In Example 8, another way of measuring ferrocyanide is shown. In this scheme, the formation of ferrocyanide is measured chemically by using 4,7 diphenyl-1,10 phenanthroline sulfonate by the method described by Avon, M. and Shavit N., Analy Biochem 6, 549 (1963). The Avon method produced color which was measured at 535 nm. The color thus produced was proportionate to the concentration of theophylline in the sample.

Example 9 illustrates the use of another electron acceptor, ferricytochrome c. In this example ferricytochrome c is reduced to ferrocyanide c. The appearance of ferrocyanide c is measured by measuring the increase in absorbance at 550 nm wavelength in the presence of the theophylline enzyme T-090. Again, the change in absorbance at 550 nm wavelength was found to be proportionate to the concentration of theophylline in the sample.
Similarly, one can use other known electron acceptors, such as nicotinamide adenine dinucleotide (NAD), 2,6-dichlorophenolindophenol (DCPIP), phenazine methosulfate (PMS), etc.

In all examples shown, one can also measure the change in reflectance as reflectance is inversely proportionate to absorbance.

The measurement of the change produced by the transferring of electrons in the above reaction is by no means limited to the spectrophotometric or reflectance methods. It is well known to use potentiometric, fluorescent or electrochemical methods to measure the transfer or change of electrons in oxidation-reduction reactions. For example, Reed and Hawkridge have shown an electron transfer reaction of cytochrome c at silver electrodes in Anal Chem 59, 2334 (1987) which can be used with this invention to measure the change of cytochrome c that occurs. Also, ferrocene or ferrocene derivatives have been used as electron acceptors for electrochemical methods as reported in the US Patent No. 4,545,382. These acceptors can also be used in the present enzymatic theophylline measurement and the change measured electrochemically. Also, the change of ferricyanide to ferrocyanide can be determined by measuring the change in current using platinum
electrodes as has been established and reported in Anal Chem 36, 343 (1964).

iii) when the enzyme is capable of demethylating theophylline by cleaving either one or both methyl groups. When one methyl group is cleaved, it produces either 1-methyl xanthine or 3-methyl xanthine along with 1 mole of formaldehyde. When both methyl groups are cleaved, it produces xanthine and 2 moles of formaldehyde. The reaction is shown below:

\[
\text{enzyme} \quad \text{theophylline + NADPH + O}_2 \rightarrow 1 \text{ methyl xanthine + formaldehyde +NADP}
\]

and/or

\[
3 \text{ methyl xanthine + formaldehyde + NADP}
\]

and/or

\[
\text{xanthine + formaldehyde + NADP}
\]

In the above reaction, NADPH is shown as electron donor. However, there are other electron donors, such as NADH or FADH, which can also be used. The formaldehyde reaction product can be measured by customary and already known chemical, enzymatic, or electrochemical methods. Example 5 shows one method of measuring formaldehyde when the
theophylline enzyme T-040 was used. As indicated in the Example, the formaldehyde thus produced was proportionate to the concentration of theophylline in the sample.

5 The reaction products xanthine or methyl xanthine can also be measured as indicated below:

\[
\text{xanthine oxidase} \\
\text{xanthine (or methyl xanthine) + O}_2 \rightarrow \text{uric acid (or methyl uric acid) + H}_2\text{O}_2
\]

The hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) formed can be measured by various methods as mentioned earlier, while uric acid or methyl uric acid can be measured, for example, by determining the increase in absorbance at 292 nm wavelength or colorimetrically as described in Clin Chem 26, 227 (1980).

20 The decrease of NADPH or NADH can also be measured spectrophotometrically or fluorometrically by customary methods as described in Anal Biochem 12, 357 (1965). The decrease of FADH can be measured by measuring the decrease at 450 nm wavelength as shown in J Biol Chem 246, 2371 (1971).
iv) other products produced by the enzymatic recognition of theophylline and measured by customary methods such as spectrophotometric, electrochemical or chromatographic or alternatively by a decrease in theophylline concentration as in Example 1.

In addition to the liquid test reactions disclosed herein, the test reagent compositions and devices of the present invention can contain state of the art additives and adjuvants which are advantageous to the reaction, such as, for example, buffers, suspending agents, thickening agents, color enhancers, surfactants, and so forth.

Moreover, in addition to the liquid reagent test systems described previously, the compositions of the present invention can advantageously be incorporated into solid carriers or matrices. Such a configuration or format is referred to in the art as dry-chemistry or solid state test device formats. The most common matrix is paper; however, other bibulous materials such as polymers, clays, gels and so forth may be utilized. Basically the reagent composition is incorporated or impregnated into the matrix and dried. In use, the device is either dipped into or contacted with the sample being tested. The signal generated in the device by the reaction of theophylline with the test composition containing inter alia the theophylline utilizing enzyme can then be detected and quantified using
state of the art techniques, such as, for example, visual comparison to a color chart, reflectance spectrophotometry, and so forth. Example 10 shows the device resulting from impregnating a filter paper with the theophylline enzyme T-090 and cytochrome c. The change in color with increasing concentration of theophylline can be read semi-quantitatively by visual inspection or quantitatively by existing reflectance measuring instruments. Alternatively, the change in electron transfer in a solid matrix can be measured electrochemically.

In summary, all the examples mentioned above and described below show that this enzymatic approach for the determination of theophylline allows the production of an easy and convenient test format not only in a liquid system but also in a solid matrix test device.
EXAMPLES

5  The following examples are intended for illustration of the present invention and are not intended to limit the scope thereof.

In all the examples given herein, the enzyme activity is defined as 1 μmole of theophylline utilized per minute at 30° C. temperature.

EXAMPLE 1

15  The assay mixture contained 0.05 M potassium phosphate buffer pH 7.0 and 0.4 u/ml of the theophylline enzyme T-060. To 2 ml assay mixture, 100 μl of a sample containing theophylline at several concentrations was added in separate cuvettes. The reaction was carried out in a Gilford spectrophotometer with 10 mm light path cuvette at 30° C. A decrease in optical density at 272 nm was observed after 30 minutes which was proportionate to the theophylline concentration in the sample.
Theophylline concentration  Decrease in OD at 272 nm

5 40 mg/L  0.067
20 mg/L  0.033
10 mg/L  0.017

Example 2

The assay mixture contained 50 μmoles/ml potassium phosphate buffer at pH 7.5 and 1.8 u/ml of the theophylline enzyme T-090 and 25 nmoles/ml of cytochrome c. To 0.5 ml assay mixture, 25 μl of a sample containing theophylline at the following concentrations were added in separate cuvettes. The reaction was carried out in a Gilford spectrophotometer with 10 mm light path cuvette at 30° C. After 20 minutes, the decrease in optical density at 272 nm was recorded which was proportionate to the theophylline concentration in the sample.
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<tr>
<th>Theophylline Concentration</th>
<th>Decrease in OD at 272 nm</th>
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<td>40 mg/L</td>
<td>0.065</td>
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<tr>
<td>20 mg/L</td>
<td>0.031</td>
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<tr>
<td>10 mg/L</td>
<td>0.015</td>
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**Example 3**

In this example, the theophylline enzyme T-090 was used. As shown in the Figure, Curve A describes the absorbance spectra of the theophylline enzyme T-090 at a concentration of 1.8 u/ml in 0.05 M potassium phosphate buffer at pH 7.5. Curve B shows the absorbance spectra of the same enzyme in the presence of theophylline at the concentration of 2 mg/L under the same conditions.

As can be seen, theophylline caused the increase in absorbance at 417.5 and 550 nm wavelength. These changes in absorptions are used as a basis for the determination of theophylline concentration in a sample.
Example 4

In this example, the theophylline enzyme T-090 was used with potassium ferricyanide as an acceptor. The 5 potassium ferricyanide changed to ferrocyanide in the presence of the enzyme when theophylline was added. The formation of ferrocyanide can be measured by measuring the decrease in optical density at 410 nm.

The assay mixture contained 50 μmoles/ml of potassium phosphate buffer, 1.8 u/ml of theophylline enzyme, and 1.43 μmoles/ml of potassium ferricyanide. To 0.35 ml assay mixture, 50 μl of a sample containing theophylline at the following concentrations was added in separate cuvettes. The reaction was carried out in a Gilford spectrophotometer with 10 mm light path cuvette at 30º C. After 30 minutes, the decrease in optical density at 410 nm wavelength was measured which was proportionate to the concentration of theophylline in the sample.
Theophylline Concentration | Decrease in OD at 410 nm
---------------------------|-----------------------
40 mg/L                    | 0.141                 
30 mg/L                    | 0.109                 
5                          |                       
20 mg/L                    | 0.076                 
10 mg/L                    | 0.047                 

Example 5

In this example, the theophylline enzyme T-040 was used. In the presence of NADPH or NADH, this enzyme demethylated theophylline and produced xanthine and/or 1 and/or 3 methyl xanthine and formaldehyde. The formaldehyde was measured by a known chemical method as reported by Nash, Biochem J 55, 416-421 (1953). The formaldehyde production was proportionate to the concentration of theophylline in the sample.

The assay mixture contained 50 μmoles/ml of Tris-HCL buffer pH 8.0, 1 μmole/ml of NADPH and 10 nmoles/ml of semicarbazide. To 2.0 ml of assay mixture in a test tube, 2.5 units of the theophylline enzyme T-040 was added. After the reaction mixture was shaken at 30°C for 15 minutes, the reaction was stopped by adding 0.6 ml of 20% zinc sulfate, 0.66 ml of saturated barium hydroxide and allowing to stand 10 minutes at room temperature. The tubes were centrifuged
at 8,000 g for 10 minutes. To 1.0 ml of supernatant, the following additions were made: 0.4 ml of Nash reagent (150 g ammonium acetate and 2 ml acetyl acetone in 500 ml of deionized water) and the tubes incubated at 60°C in a water bath for 30 minutes. Absorbance was immediately read at 415 nm wavelength.

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<td>180 mg/L</td>
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<td>40 mg/L</td>
<td>0.269</td>
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<tr>
<td>20 mg/L</td>
<td>0.140</td>
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<tr>
<td>10 mg/L</td>
<td>0.075</td>
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Example 6

In this example, the theophylline enzyme T-060 was used which produced 1,3 dimethyl uric acid and hydrogen peroxide in the presence of theophylline. The hydrogen peroxide was thus measured by a known modified Trinder's method as described by Fossati et al., Clin Chem 26, 227 (1980).

The assay mixture contained 50 μmoles/ml potassium phosphate, pH 7.5, 5 μmoles/ml of 3,5-dichloro-2-hydroxy benzene sulfonate hydrochloride (DHBS), 1 μmole/ml 4-aminoantipyrine, 5.0 u/ml of horseradish peroxidase and
0.7 u/ml of the theophylline enzyme T-060. To 0.7 ml assay mixture, 50 μl of a sample containing theophylline at the following concentrations was added in separate cuvettes. The reaction was carried out in a Gilford spectrophotometer 5 with 10 mm light path cuvette at 37° C. After 20 minutes, the increase in optical density at 510 nm was measured.

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<th>Theophylline Concentration</th>
<th>Increase in OD at 510 nm</th>
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<td>10 mg/L</td>
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<tr>
<td>20 mg/L</td>
<td>0.089</td>
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<tr>
<td>10 mg/L</td>
<td>0.045</td>
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</table>
Example 7

In this example, the theophylline enzyme T-090 was used with cytochrome c. In the presence of theophylline the reaction produced 1,3 dimethyl uric acid. This product was measured at 292 nm which is the wavelength of maximum absorbance for 1,3 dimethyl uric acid.

The assay mixture contained 50 µmoles/ml potassium phosphate buffer at pH 7.5, 1.8 u/ml of the theophylline enzyme T-090 and 25 nmoles/ml of cytochrome c. To 0.5 ml assay mixture, 25 µl of a sample containing theophylline at the following concentrations was added in separate cuvettes. The reaction was carried out in a Gilford spectrophotometer with 10 mm light path cuvette at 30° C. After 20 minutes, the increase in optical density at 292 nm was recorded which was proportionate to the theophylline concentration in the sample.
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<th>Theophylline Concentration</th>
<th>Increase in OD at 292 nm</th>
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<tr>
<td>40 mg/L</td>
<td>0.162</td>
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<tr>
<td>30 mg/L</td>
<td>0.125</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>0.087</td>
</tr>
<tr>
<td>10 mg/L</td>
<td>0.036</td>
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**Example 8**

In this example, the theophylline enzyme T-090 was used with potassium ferricyanide as an acceptor which produces potassium ferrocyanide in the presence of theophylline. The ferrocyanide thus produced is measured chemically by using 4,7 diphenyl-1,10 phenanthroline sulfonate as described by Avon and Shavit in Anal Biochem 6, 549 (1963).

The assay mixture contained 50 µmoles/ml of potassium phosphate buffer at pH 7.5, 1.8 u/ml of the theophylline enzyme T-090, and 1.43 µmoles/ml of potassium ferricyanide. To 0.35 ml assay mixture, 50 µl of a sample containing theophylline at the following concentrations was added. The reaction was carried out in a Gilford spectrophotometer with 10 mm light path cuvette at 30° C for 30 minutes. From the above, 50 µl of assay mixture was mixed with 35 µl of deionized water and 150 µl of color producing reagent. The color producing reagent contains 1 M sodium acetate, 0.066 M
citric acid, .00055 M ferrichloride in 0.1 M acetic acid, and 83.3 μg of 4,7-diphenyl-1,10 phenanthroline. After 6 minutes the absorbance was measured at 535 nm wavelength. The absorbance is proportionate to the concentration of theophylline.

<table>
<thead>
<tr>
<th>Theophylline Concentration</th>
<th>Increase in OD at 535 nm</th>
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<tbody>
<tr>
<td>40 mg/L</td>
<td>0.319</td>
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<tr>
<td>30 mg/L</td>
<td>0.241</td>
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<td>20 mg/L</td>
<td>0.171</td>
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<td>10 mg/L</td>
<td>0.080</td>
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<tr>
<td>5 mg/L</td>
<td>0.045</td>
</tr>
</tbody>
</table>

Example 9

In this example, the theophylline enzyme T-090 was used with ferricytochrome c as an acceptor which produces 1,3 dimethyl uric acid and ferrocytochrome c in the presence of theophylline. The formation of ferrocytochrome c is measured by the increase in absorbance at 550 nm wavelength.

The assay mixture contained 50 μmoles/ml of potassium phosphate buffer at pH 7.5, 5 u/ml of the theophylline enzyme T-090, and 0.25 nmole/ml horse ferricytochrome c.
To 0.5 ml assay mixture, 25 μl of sample containing theophylline at the following concentrations was added in separate cuvettes. The reaction was carried out in a Gilford spectrophotometer with 10 mm light path cuvette at 5 30° C. After 15 minutes, when the reaction was complete, the increase in optical density was measured. As in the other examples, the absorbance has a linear relationship to the concentration of theophylline in the sample.

<table>
<thead>
<tr>
<th>Theophylline Concentration</th>
<th>Increase in OD at 550 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 mg/L</td>
<td>0.454</td>
</tr>
<tr>
<td>30 mg/L</td>
<td>0.361</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>0.264</td>
</tr>
<tr>
<td>15</td>
<td></td>
</tr>
<tr>
<td>10 mg/L</td>
<td>0.109</td>
</tr>
<tr>
<td>5 mg/L</td>
<td>0.055</td>
</tr>
</tbody>
</table>

**Example 10**

Ten by ten mm square filter paper was impregnated with a solution containing the theophylline enzyme T-090 at various concentrations. For example, 50 u of the theophylline enzyme T-090 in 0.05 M phosphate buffer, pH 7.5. This solution also contained 1 μmole/ml of ferricytochrome c. The paper was dipped in the above solution and air dried. When 50 μl of serum containing different concentrations of
theophylline, 5-40 mg/L, was added to the filter paper, increasingly deeper shades of pink appeared corresponding to the increasing theophylline concentration. The gradation of pink color allowed the estimation of the different 5 theophylline concentrations.
CLAIMS

1. A method for determining theophylline in a sample which comprises contacting the sample with a theophylline utilizing enzyme which provides a measurable signal in the presence of theophylline, determining the amount of signal produced and correlating this amount to the concentration of theophylline in the sample.

2. A method according to claim 1 in which said sample is selected from the group consisting of body fluids, food extracts and medicinal compositions.

3. A method according to claim 1 in which the signal is generated as a result of a decrease in the theophylline present in the sample upon contact with the enzyme.

4. A method according to claim 3 in which the signal is measured by observing a change in the optical density of the sample at a wavelength of from about 260 nm to about 280 nm.

5. A method according to claim 1 in which the signal is a result of a change in the enzyme upon contact with theophylline present in the sample.
6. A method according to claim 5 in which the change is an increase in optical density of the sample at a wavelength in the range of from about 410 nm to about 550 nm.

7. A method according to claim 1 wherein an electron carrier is present.

8. A method according to claim 7 in which the signal is generated as a result of a decrease in the theophylline present in the sample upon contact with the enzyme.

9. A method according to claim 8 in which the signal is measured by observing a change in the optical density of the sample at a wavelength of from about 260 nm to about 280 nm.

10. A method according to claim 7 in which the signal is a result of a change in the enzyme upon contact with theophylline present in the sample.

11. A method according to claim 10 in which the change is an increase in optical density of the sample at a wavelength in the range of from about 410 nm to about 550 nm.

12. A method according to claim 10 in which the change is measured electrochemically.
13. A method according to claim 7 in which the electron carrier is an electron donor.

14. A method according to claim 13 in which the electron donor is selected from the group consisting of reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate and reduced flavine adenine dinucleotide.

15. A method according to claim 7 in which the electron carrier is an electron acceptor.

16. A method according to claim 15 in which the electron acceptor is oxygen.

17. A method according to claim 15 in which the electron acceptor is selected from the group consisting of cytochromes, ferricyanides, dichlorophenolindophenol, nicotinamide adenine dinucleotide, phenazine methosulfate and methylene blue.

18. A method according to claim 1 in which a reaction product caused by the interaction of the theophylline utilizing enzyme and theophylline present in the sample generates the measurable signal.
19. A method according to claim 18 in which the reaction product is 1,3-dimethyl uric acid.

20. A method according to claim 19 in which the 1,3-dimethyl uric acid is determined by measuring the increase in the optical density of the sample at a wavelength in the range of from about 285 to about 305 nm.

21. A method according to claim 18 in which hydrogen peroxide is produced as a reaction product and is the measurable signal.

22. A method according to claim 21 in which the hydrogen peroxide is measured by a system consisting essentially of a chromogenic reagent which is capable of undergoing a color change in the presence of hydrogen peroxide.

23. A method according to claim 7 in which a change in the electron carrier produces the measurable signal.

24. A method according to claim 7 in which the electron carrier is nicotinamide adenine dinucleotide and produces NADH which is the measurable signal.

25. A method according to claim 7 in which the electron carrier is ferricytochrome c and ferrocytochrome c is produced as the measurable signal.
26. A method according to claim 7 in which the electron carrier is potassium ferricyanide which produces potassium ferrocyanide as the measurable signal.

27. A method according to claim 7 in which the electron carrier is flavin adenine nucleotide which is reduced to produce the measurable signal.

28. A method according to claim 7 in which the electron carrier is 2,6 dichlorophenolindophenol which is reduced to produce the measurable signal.

29. A method according to claim 18 in which the theophylline utilizing enzyme is capable of changing theophylline to a reaction product selected from the group consisting of xanthine and xanthine derivatives.

30. A method according to claim 18 in which the reaction product is determined in the presence of xanthine oxidase to produce hydrogen peroxide.

31. A method according to claim 18 in which the reaction product is measured in the presence of xanthine dehydrogenase and an electron acceptor.
32. A method according to claim 18 in which formaldehyde is produced as a reaction product and is the measurable signal.

33. A test composition for the determination of theophylline in a sample comprising a theophylline utilizing enzyme capable of producing a measurable signal in the presence of theophylline and a means for measuring and correlating the signal to the quantity of theophylline present in the sample.

34. A test composition according to claim 33 in which the signal is generated as a result of a decrease in theophylline present in the sample upon contact with the enzyme.

35. A test composition according to claim 34 in which the signal is measured by observing a change in the optical density of the sample at a wavelength of from about 260 nm to about 280 nm.

36. A test composition according to claim 33 in which the signal is a result of a change in the enzyme upon contact with theophylline present in the sample.
37. A test composition according to claim 36 in which the change is an increase in optical density of the sample at a wavelength in the range of from about 410 nm to about 550 nm.

38. A test composition according to claim 33 in which an electron carrier is present.

39. A test composition according to claim 38 in which the signal is generated as a result of a decrease in the theophylline present in the sample upon contact with the enzyme.

40. A test composition according to claim 39 in which the signal is measured by observing a change in the optical density of the sample at a wavelength in the range of from about 260 nm to about 280 nm.

41. A test composition according to claim 38 in which the signal is a result of a change in the enzyme upon contact with theophylline present in the sample.

42. A test composition according to claim 41 in which the change is an increase in the optical density of the sample at a wavelength in the range of from about 410 nm to about 550 nm.
43. A test composition according to claim 41 in which the change is measured electrochemically.

44. A test composition according to claim 38 in which the electron carrier is an electron donor.

45. A test composition according to claim 44 in which the electron donor is selected from the group consisting of reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate and reduced flavine adenine dinucleotide.

46. A test composition according to claim 38 in which the electron carrier is an electron acceptor.

47. A test composition according to claim 46 in which the electron acceptor is oxygen.

48. A test composition according to claim 46 in which the electron acceptor is selected from the group consisting of cytochromes, ferricyanides, dichlorophenolindophenol, nicotinamide adenine dinucleotide, phenazine methosulfate and methylene blue.

49. A test composition according to claim 33 in which the means is a reagent responsive to the presence of a reaction
product of the theophylline utilizing enzyme and
theophylline.

50. A test composition according to claim 49 in which the
reaction product is hydrogen peroxide.

51. A test composition according to claim 49 in which the
reagent is responsive to a reaction product selected from
the group consisting of xantine and xanthine derivatives.

52. A test composition according to claim 51 in which the
reagent comprises xanthine oxidase.

53. A test composition according to claim 50 in which the
reagent also comprises peroxidase and a oxidation-reduction
indicator.

54. A test composition according to claim 49 in which the
reagent is responsive to formaldehyde.

55. A test composition according to claim 49 in which the
reagent is responsive to 1,3-dimethyl uric acid.

56. A test composition according to claim 33 in which the
signal is measured using a means selected from the group
consisting of spectrophotometric, electrochemical,
fluorescent, reflectance and polarization methodologies.
57. A test device for the determination of theophylline in a sample comprising a solid matrix incorporated with the residue of a composition consisting essentially of a theophylline utilizing enzyme capable of producing a measurable signal in the presence of theophylline.

58. A test device according to claim 57 in which the composition additionally comprises an electron carrier.

59. A test device according to claim 58 in which the electron carrier is an electron donor.

60. A test device according to claim 59 in which the electron donor is selected from the group consisting of reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate and reduced flavine adenine dinucleotide.

61. A test device according to claim 58 in which the electron carrier is an electron acceptor.

62. A test device according to claim 61 in which the electron acceptor is oxygen.

63. A test device according to claim 61 in which the electron acceptor is selected from the group consisting of
cytochromes, ferricyanides, dichlorophenolindophenol, nicotinamide adenine dinucleotide, phenazine methosulfate and methylene blue.

64. A test device according to claim 57 in which the composition responds to a reaction product generated by the action of the theophylline utilizing enzyme on theophylline present.

65. A test device according to claim 57 in which a change in the enzyme is measured.

66. A test device according to claim 57 in which a decrease in the theophylline present is measured.

67. A test device according to claim 57 in which the solid matrix is paper.

68. A test device according to claim 57 in which the solid matrix is a polymer.

69. A test device according to claim 58 in which the solid matrix is paper.

70. A test device according to claim 58 in which the solid matrix is a polymer.
INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC(4): C12Q 1/00, C12Q 1/26, C12Q 1/32
U.S. Cl.: 435/4, 25, 26

II. FIELDS SEARCHED

Classification System Classification Symbols
U.S. 435/4, 10, 25, 26, 28, 189, 191, 192, 805, 810, 817
422/56, 57, 61

Documentation Search other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Computer Search: Medline, WPI, WPIL, CA Search, APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT 2

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of Document, 7 with indication, where appropriate, of the relevant passages 12</th>
<th>Relevant to Claim No. 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>Chemical Abstracts, Volume 107, No. 25, issued 21 December 1987 (Columbus, Ohio, USA) R.A. Robson, &quot;Characterization of Theophylline Metabolism In Human Liver Microsomes&quot;, see page 13, column 2, the abstract No. 228376r, Br. J. Clin. Pharmacol. 1987, 24(3), 293-300 (Eng).</td>
<td>1-70</td>
</tr>
<tr>
<td>Y,P</td>
<td>US, A, 4,810,640 (Nakamura) 07 March 1989, see column 8, line 24- column 9, line 6.</td>
<td>5-7,11-13</td>
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<td>15-17,21-23</td>
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<td>59,61-63</td>
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<tr>
<td>Y</td>
<td>US, A, 4,341,868 (Nakanishi) 27 July 1982, see column 3, lines 8-29.</td>
<td>30,52</td>
</tr>
</tbody>
</table>

* Special categories of cited documents: 10
 "A" document defining the general state of the art which is not considered to be of particular relevance
 "E" earlier document but published on or after the international filing date
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 "O" document referring to an oral disclosure, use, exhibition or other means
 "PP" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 25 April 1989

Date of Mailing of this International Search Report

International Searching Authority ISA/US

Signature of Authorized Officer

ESTHER M. KEPPLINGER
FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

| Y | US, A, 4,235,869 (Schwarzberg) 25 November 1980, see column 13, lines 21-24 and column 16, line 65-column 17, line 23. | 31 |
| Y | US, A, 4,216,292 (Ikuta) 05 August 1980, see column 5, lines 25-44 column 6, line 68--column 7, line 18 and column 7, lines 46-53. | 32,54 |

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers , because they relate to subject matter not required to be searched by this Authority, namely:

2. Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out specifically:

3. Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.