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[54] **INTERFERANT REMOVAL FROM AMPHETAMINE IMMUNOASSAY**

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[57] **ABSTRACT**

A method for removing β-hydroxyamine compounds as an interferant from immunoassays, where like compounds, such as amphetamine, methamphetamine, and the like are to be determined. The sample, for example, urine is treated with aqueous periodate at a mildly basic pH for a time sufficient to remove the β-hydroxyamine as an interferant, the pH being maintained by an ammonium hydroxide. The sample may then be used in assays for the determination of amphetamine or like compounds without interference from the β-hydroxyamine compound.

10 Claims, No Drawings

INTERFERANT REMOVAL FROM AMPHETAMINE IMMUNOASSAY

BACKGROUND OF THE INVENTION

1. Field of the Invention

There is frequent need for accurate and reliable determination of amphetamine, methamphetamine or like materials. Since amphetamine is a drug of abuse, there is the police function of determining whether a person has taken amphetamine. There is also the medicinal function of determining the amount of amphetamine in a biological fluid.

Phenylpropanolamine is a common drug sold over the counter, which is taken in relatively large dosages. Because of the relatively high concentration of the phenylpropanolamine in biological fluids when used and the structural similarity of the phenylpropanolamine to amphetamine, the phenylpropanolamine obscures the detection of the amphetamine, for example, in chromatography or by providing a false signal, as in immunoassays.

Immunoassays are based on employing a receptor, usually an antibody, which is capable of recognizing or distinguishing a particular chemical structure. By employing techniques which distinguish between those compounds which bind to the receptor and those compounds which do not, the compound recognized by the receptor can be determined, qualitatively or quantitatively.

Radioimmunoassays which allow for a competition between a radioactively labelled compound and the compound to be assayed have been known for a long time. More recently, two new and unique methods have been developed. The first method employs an electron spin resonance technique, entitled FRAT, supplied by Syva Corp. The second technique employs enzyme labelled with a compound which simulates the compound to be assayed, and is called EMIT™, also supplied by Syva Corp. The accuracy of these techniques is dependent upon, among other variables, the ability for the receptor - the antibody - to recognize a specific compound. In preparing antibodies to a specific compound, the compound is bonded to an antigen and the antigen injected in an animal. This results in the production of antibodies which recognize the compound. Compounds which have this property are referred to as haptens.

While antibodies show high specificity for particular geometry or spatial configuration and a particular distribution of polar and non-polar groups, antibodies will frequently recognize similar compounds to a lesser degree and infrequently to a greater degree. This recognition of compounds other than the compound of interest is referred to as cross-reactivity.

Where there is undesirable cross-reactivity, and the cross-reacting compound is sufficiently prevalent in the samples to be determined, so as to be of concern, it is necessary to find some way to remove or change the compound, so that the antibody will not recognize it or bind the compound. It is found that with derivatives of β-phenethyl amine, e.g., amphetamine and methamphetamine, β-hydroxyphenethyl amine compounds are strong interferants. Since compounds such as phenylpropanolamine are frequently in decongestants sold over the counter, it is necessary to be able to distinguish between amphetamine, for example, and phenylpropanolamine. The method employed for allowing the

antibody to distinguish between the two must not interfere with the assay system. Furthermore, it must not affect the phenethylamine derivative, so as to modify the response of the antibody to the phenethylamine derivative. Nor may the method chosen affect the label, so as to give a spurious result.

2. Description of the Prior Art

A description of the free radical assay technique can be found in U.S. Pat. No. 3,690,834, issued Sept. 12, 1972. A description of the enzyme technique may be found in copending application Ser. No. 143,609, filed May 14, 1971, now abandoned. A description of the radioimmunoassay technique may be found in numerous texts, such as Kirkham, et al., Radioimmunoassay Methods, European Workshop, September 1970, Churchill, London 1971; Ferrari, Gazz. Chim. Ital. 92 22 (1962) reports the formation of crystalline tetramethylammonium periodate.

SUMMARY OF THE INVENTION

A medium to be assayed for a phenethylamine derivative is combined with a small amount of an aqueous periodate solution at a mild temperature and mildly basic pH for a sufficient time to modify any β-hydroxy-β-phenethylamine derivative, which acts as an interferant, so as to destroy its interfering effect. The sample is then used in the determination without interference from the β-hydroxyamine. The method finds particular use with immunoassays.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Liquid media suspected of containing a β-phenethylamine derivative, such as amphetamine or methamphetamine are treated with a small amount of periodate at a mildly basic pH (8) for a sufficient time to modify any aralkyl β-phenethylamines, so as to prevent obtaining a spurious result because of the presence of phenylpropanolamine or the like compound. That is, those compounds having vicinal hydroxyamines and an aliphatic group bonded to a phenyl ring. These interferants are normally of from eight to 10 carbon atoms.

The sample may then be used in a normal way in any of the variety of determinations, such as thin layer chromatography, immunoassays, e.g., radioimmunoassay, the electron spin resonance technique, referred to as FRAT or the enzyme technique, referred to as EMIT™. The immunoassays will usually be carried out at a pH below 8, usually in the range of 5.0 to 7.5, more usually 5.5 to 7.0.

The medium may be any source suspected of containing the phenethylamine. Of particular interest are physiological media, such as blood serum, saliva, and urine, particularly urine. Any of the normal treatments given to the fluid may be carried out prior to the periodate treatment. When the medium has been properly prepared, it is combined with the periodate at a mildly basic pH and allowed to stand, with mild agitation if desired, for a sufficient time to modify or destroy the interfering substances sensitive to the periodate treatment.

The amount of periodate will vary depending on the particular medium and the suspected degree of contamination with interferants, but will normally be in the range of about 10^{-3} to 10^{-7} moles/ml, and more usually about 10^{-4} to 10^{-6} moles/ml, and particularly about 10^{-5} moles/ml.

The basic pH, usually in the range of 8 to 10, can be achieved with a variety of bases. The periodate forms complex ions with varying pH and these complex ions are of varying solubility in aqueous systems. Conveniently, ammonium hydroxide (ammonium includes quaternary ammonium hydroxides of from 1 to 12, usually one to six carbon atoms) is used to achieve the desired pH. Preferred hydroxides are ammonium hydroxide (NH_4OH) and tetraalkyl ammonium hydroxide of from four to eight carbon atoms. The ammonium hydroxide will be present in amounts sufficient to provide a pH of the treated medium in the range of about 8 to 10 usually 8 to 9. Usually the amount in moles of ammonium hydroxide will be 2 to 20 times that of moles of periodate, more usually from about 5 to 15 times that of the periodate. Conveniently, a reagent solution can be prepared which is 0.01 to 0.5, more usually 0.05 to 0.3 M in periodate and 0.25 to 2, more usually 0.5 to 1.5 M in ammonium hydroxide.

It is found that at concentrations of periodate useful as reagents for combining with physiological fluids, short shelf lives are obtained with the usual alkali metal cations at the basic pH. With tetraalkylammonium cations, long shelf lives are obtained, so that the reagents can be prepared, stored and shipped without any precipitate forming.

The temperature of the periodate treatment will normally be in the range of about 0° to 40°C, more usually from about 10° to 30°C, and the assays can be conveniently carried out at ambient temperatures. The time for treatment will usually be as short as possible to obtain the desired results and will usually be from about 1 to 10 minutes, more usually about 5 minutes.

The periodate is conveniently employed as an alkali metal periodate, particularly sodium and potassium, and preferably sodium. The quaternary ammonium hydroxides are illustrated by tetraethylammonium hydroxide, diethyldimethylammonium hydroxide, N,N-dimethyl piperidinium hydroxide, N-methyl pyridinium hydroxide, and the like. Usually, the quaternary ammonium hydroxide will be free of unsaturation, both aliphatic and aromatic.

After treatment with the periodate, the sample is ready to be used. In immunoassays, the assay solution is normally sufficiently buffered to control the pH. The immunoassay solution will normally be at a pH of 7.5 or below. At these pHs, the periodate is found not to adversely affect the various reagents or interfere with the immunoassay determination.

In other types of determination, e.g., chromatography — thin layer chromatography — the sample may be used directly.

To demonstrate the subject invention, a number of urine samples were tested, employing the enzyme assay system. In carrying out the test, 20μl of a reagent which was 0.13 M in sodium periodate and 1.2 F in tetramethylammonium hydroxide was combined with urine (100 μl) and allowed to stand for five minutes.

In carrying out the assay, N-carboxymethylamphetamine was employed for determining the amphetamine present. N-carboxymethylamphetamine was conjugated with lysozyme as follows:

(All temperatures are in Centigrade)

N-carboxymethylamphetamine (25 mg, 0.133mM) was suspended in 1.8 ml of dry dioxane at 40°. Phosgene (12.5 volume percent in benzene) (0.715 ml) was added in one portion. The reaction mixture was stirred at 40° for 3.5 hours before an additional 0.2 ml of phosgene (12.5 volume percent in benzene) was added. After stirring an additional 30 minutes, the solution became homogeneous. The solvent was removed in vacuo at 25° in the hood. An additional 0.70 ml of dry dioxane was added for use in the next step.

The cold dioxane solution of the above product was added dropwise over 5 minutes to a stirred, cold (0°) solution of 200 mg sodium bicarbonate and 100 mg lysozyme in 10 ml water. The milky reaction mixture was stirred at 4° for 48 hours and dialyzed against 1.1 changed 3 times daily over a 48 hour period. The residue was lyophilized.

The assay is carried out by combining 0.2 ml of M. luteus (30 mg) in 50 ml of 0.05 M Tris-maleate of pH 6 and 20λ of amphetamine antibody (1.3×10^{-5}M in binding sites), followed by the addition of 80λ of the urine sample. The mixture was then diluted with 0.5 ml of a solution of the above modified enzyme to provide a ratio of binding sites to moles of lysozyme of 1.5:1. The reaction mixture was then aspirated into the spectrometer and the decrease in optical density measured at 436 nm for 40 seconds at 30°.

A standard urine sample was employed to which a variety of different materials were added, and the results determined with and without the periodate treatment. The following table indicates the results:

TABLE I

		RATE	
		Without IO_4 Treatment	With IO_4 Treatment
URINE		46	47
1.0	μg/ml amphetamine	64	55
5.0	μg/ml amphetamine	95	85
50.0	μg/ml amphetamine	173	152
50	μg/ml ephedrine	117	45
100	μg/ml ephedrine	132	47
50	μg/ml phenylpropanolamine	99	46
100	μg/ml phenylpropanolamine	137	46

The periodate is very effective in removing phenylpropanolamine and ephedrine as an interferant. While the result for amphetamine changes with periodate treatment, a different standard curve can be obtained, which is reproducible, so that the same results for the amphetamine can be obtained with and without periodate treatment by using a different standard curve, where an interferant is not present.

Samples of lyophylized urine containing 0.0 and 1.0 μg/ml of amphetamine were employed. Employing the assay technique described above, the 0.0 sample gave readings of 48; 50; 50 OD/min, and the 1.0 μg/ml sample gave readings of 59; 61 OD/min. When 200 μl of aqueous sample was combined with 10 μl of 0.5 g/ml sodium periodate and 10 μl ammonium hydroxide (10 wt percent in water) the results for the two samples were: 0.0 μg/ml-48; 50; and 1.0 μg/ml-62; 57. When aqueous phenylpropanolamine at a concentration of 55 μg/ml was employed the reading was 70; 81 OD/min. After treatment of the sample with the ammonium hydroxide and periodate and waiting five minutes, the readings were 52; 52 OD/min.

The further demonstrate the effect of the sodium periodate, 100 μl of the 1.0 μg/ml amphetamine solution was combined with 15 μl of 0.5 g/10 ml aqueous so-

dium periodate and 5 μl of 10 wt percent aqueous sodium hydroxide. The reading was 60 OD/min after 2 minutes. When the determination was repeated replacing the 100 μl of 1.0 μg aqueous amphetamine solution with 100 μl aqueous phenylpropanolamine solution (55 μg/ml) the readings were 41; 46 OD/min. This is equivalent to background.

A series of urine samples were obtained which were verified to contain amphetamine by gas-liquid chromatography. The samples were then tested according to the above procedure by the enzyme techniques, as well as by the electron spin resonance technique.

In the FRAT assay, N'-2',2',5',5'-tetramethylpyrrolidinyl-1'-oxyl 1-phenyl-2-propylaminoacetamide is the spin label reagent and is employed at a concentration of 2.85×10^{-6} M, with the mole ratio of spin label to antibody (based on binding sites) of 0.95. The concentration of borate buffer at pH 8.0 is 0.18 M. The sample is treated with a small amount of dichromate to destroy any ascorbic acid and 20 μl of sample is added to 5 μl of antibody solution and 5 μl of the spin label at the appropriate buffer concentration. The sample is then taken up in an ESR capillary tube and read in an ESR spectrometer and the amphetamine concentration determined by comparison with a standard.

The following table indicates the results.

TABLE II

GLC Result μg/ml		FRAT* μg/ml	EMIT* μg/ml Without IO_4	μg/ml With IO_4
Amphetamine	Methamphetamine			
16		9.6	6.7	8.0
1	18	8.7	6.7	6.5
14		41	35	34
9	400–700	35	34	>50
5	5	3.8	3.8	5.7
3		14	8.3	10.2
9		18.5	9.4	8.8
14		19.5	14.0	15.0
1	24	33	27.5	32.0
21	37	1.95	2.6	3.0
4		3.7	3.0	3.1
45		>100	>50	>50
550		>100	>50	>50

*Total of amphetamine and methamphetamine

The above results show that the periodate does not affect the results obtained with amphetamine. Therefore, the amphetamine can be accurately assayed for by removing interferants such as phenylpropanolamine without affecting the assay for the β-phenethylamine derivative.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the invention, as limited only by the scope of the appended claims.

What is claimed is:

1. In an assay method for determining aralkyl amines, wherein 1-aralkyl β-hydroxyamines interfere, the improvement which comprises: treating the sample to be assayed at a pH greater than about 8 with an amount of aqueous periodate solution sufficient to remove the hydroxyamine interferant wherein said pH is maintained by the presence of an ammonium hydroxide.

2. A method according to claim **1**, wherein the concentration of said periodate is in the range of about 10^{-3} to 10^{-7} moles/ml.

3. A method according to claim **1**, wherein said periodate is at a concentration in the range of about 10^{-4} to 10^{-6} moles/ml.

4. A method according to claim **1**, wherein the concentration of said ammonium hydroxide is in an amount of from 2 to 20 times the concentration of periodate and said ammonium hydroxide is tetraalkyl ammonium hydroxide.

5. In a method for carrying out immunoassays to detect amphetamine or methamphetamine, the improvement which comprises: treating the sample to be assayed with aqueous periodate solution at a concentration in the range of about 10^{-4} to 10^{-6} moles/ml in the presence of an ammonium hydroxide in an amount sufficient to provide a pH in the range of about 8 to 9.

6. A method according to claim **5**, wherein said ammonium hydroxide is tetramethyl ammonium hydroxide.

7. A method according to claim **5**, wherein said ammonium hydroxide is NH_4OH.

8. A method according to claim **5**, wherein said treating is carried out at a temperature in the range of about 10° to 40° C and for a time in the range of about 1 to 10 minutes.

9. An aqueous solution which is 0.05 to 0.5 M in periodate and 0.5 to 2 M in tetraalkylammonium hydroxide of from four to eight carbon atoms.

10. A solution according to claim **9**, wherein said tetraalkylammonium hydroxide is tetramethylammonium hydroxide and said periodate ranges from 0.05 to 0.3 M in concentration.

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