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^{125}I Radioimmunoassay for the Dual Detection of Amphetamine and Methamphetamine

REFERENCE: Ward, C., McNally, A. J., Rusyniak, D., and Salamone, S. J., "**^{125}I Radioimmunoassay for the Dual Detection of Amphetamine and Methamphetamine,**" *Journal of Forensic Sciences,* JFSCA, Vol. 39, No. 6, November 1994, pp. 1486–1496.

ABSTRACT: A radioimmunoassay that exhibits a nearly equivalent response to D-amphetamine and D-methamphetamine in urine over the assay range of 0 to 1000 ng/mL while displaying low cross-reactivity to L-amphetamine and L-methamphetamine (4.6% and 2.4%, respectively) has been developed. In addition, methylenedioxy-amphetamine (MDA) and methylenedioxymethamphetamine (MDMA) were detectable in the assay with cross-reactivity levels of >100% and 77% respectively. Little cross-reactivity was observed with the commonly encountered over-the-counter (OTC) drugs and this cross-reactivity was further reduced by the addition of sodium periodate into the reaction mixture to oxidize the β-hydroxylamines. The double (second) antibody assay uses ^{125}I-radiolabeled derivatives of both D-amphetamine and D-methamphetamine as tracers in combination with two highly specific sheep antisera directed against D-amphetamine and D-methamphetamine. The assay exhibits a dose-response of approximately 90,000 dpm from 0 to 1000 ng/mL of D-amphetamine or D-methamphetamine with a minimum detectable dose for either drug of approximately 25 ng/mL. With a cut-off level of 500 ng/mL, the assay gave a positive result for 100% of the 111 clinical samples containing GC/MS confirmed (at or above the NIDA GC/MS cut-off values) levels of amphetamine and/or methamphetamine. Eighty-eight samples that screened negative in a clinical laboratory were all negative in the assay. Nineteen samples which were incorrectly identified as positive by other commercially available amphetamine assays were negative in this RIA.

KEYWORDS: toxicology, amphetamines, RIA, detection of drugs

The amphetamines, which includes both amphetamine and methamphetamine, are a major class of central nervous system (CNS) stimulants related chemically and pharmacologically to the naturally occurring human catecholamines, epinephrine and norepinephrine. These drugs cause decreased appetite, increased wakefulness, and at times cause a sense of increased energy, self-confidence, and euphoria [*1,2*]. In addition as CNS stimulants, their effects may include agitation, tremors, and increased motor activity [*1–3*]. For these reasons the amphetamines have become widely abused and are included in most broad-based abused drug screening programs, both in the civilian and military environments. The many issues complicating the analysis of this drug class have been discussed in numerous recent publications [*4–9*]. These include: The existence of legally obtainable isomeric forms of the drugs;

Received for publication 10 Jan. 1994; revised manuscript received 6 April 1994; accepted for publication 8 April 1994.

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the presence of structurally related compounds occurring both naturally, as mentioned, and in over-the-counter medications; and problems associated with changing guidelines issued by the National Institute on Drug Abuse (NIDA, now The Substance Abuse and Mental Health Services Administration) with respect to amphetamines confirmation. Attempts to develop an assay for amphetamines that could satisfactorily address these issues in a clinical laboratory setting have not been entirely successful. Amphetamines testing is viewed, therefore, as one of the more problematic areas in clinical toxicology.

The immunoassays currently available to detect amphetamines can be considered to be of three types: 1. Those assays that are highly specific for either amphetamine and its designer drug counterpart, methylenedioxyamphetamine (MDA); or methamphetamine and its designer drug counterpart, methylenedioxymethamphetamine (MDMA), but not both sets simultaneously. These assays generally exhibit low cross-reactivity to the structurally related compounds. Examples of these assays are offered by Roche Diagnostic Systems, and Diagnostic Products Corporation. Both companies market separate radioimmunoassays (RIA) for amphetamine and methamphetamine which fall into this category. 2. The second type of immunoassay available consists of those that are able to detect both amphetamine and methamphetamine to some varying extent, but which also exhibit higher levels of cross-reactivity to the β-hydroxylamine compounds or to the legal isomers of the amphetamines. The Syva enzyme immunoassays (EIA) and the Abbott fluorescence polarization assay are examples of this type. The Syva assays display varying levels of cross-reactivity to the OTC compounds, whereas the Abbott assay has higher cross-reactivity to L-amphetamine. 3. The third type of assay available today is represented by Roche Diagnostic Systems' ONLINE® assay. This assay displays low levels of cross-reactivity to the OTC compounds and is a dual assay for the detection of both amphetamine and methamphetamine. It was, however, designed to parallel the present NIDA guidelines for the GC/MS confirmation of amphetamines and detects methamphetamine only when amphetamine is also present.

The RIA described here represents an alternative approach to amphetamines screening. This approach uses two highly specific single antigen assays combined into one system that allows for the equal detection of either amphetamine or methamphetamine and/or an enhanced detection of amphetamine and methamphetamine together. The approach is similar to that of the new Emit II amphetamines assay but offers one distinct advantage, the elimination of cross-reactivity to the β-hydroxylamines. This lack of cross-reactivity is accomplished by the use of sodium periodate for the oxidation of β-hydroxylamines during the RIA incubation. The assay thus detects both amphetamine and methamphetamine and eliminates false positive problems associated with β-hydroxylamines.

Materials and Methods

Reagents

Amphetamine·H_2SO_4 methamphetamine·HCl, ephedrine·HCl, pseudoephedrine·HCl, norpseudoephedrine·HCl, phenylpropanolamine·HCl, and sodium (m) periodate were obtained from Sigma. Sodium ^{125}iodide was obtained from Amersham; PIC B8 reagent was obtained from Millipore; HPLC grade methanol and water, and Triton x-100 were obtained from Fisher. Bovine serum albumin (BSA) was obtained from Miles and donkey anti-goat IgG serum was obtained from Lampire Biologicals. FD&C yellow #5 and blue #1 were obtained from Warner Jenkensen. Polyethylene glycol (PEG) was obtained from Van, Waters, and Rogers. Iodination and immunization derivatives were synthesized in-house.

Solutions

Tracer Diluent—consisted of 25% ethanol in a buffer containing 10 mM sodium and potassium phosphate, 0.9% sodium chloride, 0.1% sodium azide, 0.05% triton X-100, and 0.01% FD&C yellow #5, pH 7.2.

Antibody Diluent—consisted of 10 mM sodium and potassium phosphate, 0.9% sodium chloride, 0.5% BSA, 0.2% sodium azide, and 0.01% FD&C blue #1, pH 7.2.

Second Antibody Solution—consisted of 10 mM sodium and potassium phosphate, 0.9% sodium chloride, 5% donkey anti-goat IgG serum, 4% PEG, 0.1% sodium azide, 0.01% FD&C yellow #5, and 0.01% FD&C blue #1, pH 7.2.

Sodium Periodate Solution—consisted of 100 mM sodium periodate in 25 mM sodium acetate with 0.1% sodium azide, pH 4.5.

Preparation of ^{125}I Radiolabeled Amphetamine and Methamphetamine Tracer Solution

Radiolabeling of the amphetamine and methamphetamine derivatives was performed using a modification of the Greenwood, Hunter procedure (10): To 5 mCi carrier free sodium ^{125}iodide was added 50 μL of a 1 mg/mL methanolic solution of either (S)-N-[2-(4-hydroxyphenyl)-ethyl]-4-(2 aminopropyl) benzene-butanamide or (S)-N-[2-(4 hydroxyphenyl)-ethyl]-4[2-(methylamino)propyl] benzenebutanamide (Fig. 1 structures 1 and 2, respectively). After mixing, 50 μL of freshly prepared 5 mg/mL chloramine T in 25% methanol, 75% 50 mM sodium borate buffer, pH 8.4 was added to a final volume of 150 μL. The solution was then mixed gently for 90 s. The reaction was then stopped with the addition of 50 μL of 10 mg/mL sodium bisulfite in 25% methanol: 75% 50 mM sodium borate buffer, pH 8.4. These reaction mixtures were then separately purified by HPLC.

HPLC Purification of the Radiolabeled Tracers

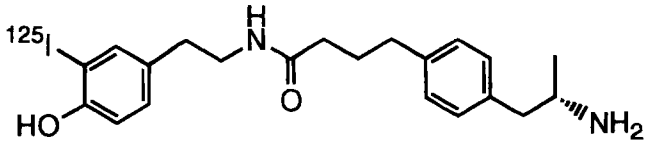
The entire reaction volume was added to a 25 cm × 10 mm C_{18} column (5 μ ODS Beckman) previously equilibrated with a 50:50 mixture of methanol and water containing one vial of PIC B8 reagent per liter. Three mL fractions were collected at a flow rate of three mL/minute and monitored at 258 nm; the fractions were then counted and the radiolabeled drug fractions were pooled. Aliquots of both pools were then separately diluted with tracer diluent to 411 000–437 000 dpm/200 μL and adjusted as necessary within that range until the amount of radioactivity in the two pools matched to within 2%. The two tracer solutions were then blended in a ratio of 1 part methamphetamine tracer to 1.1 × parts amphetamine tracer.

Production of Sheep Antiserum

Immunogens were (S)-N-[4-[4-(2-Aminopropyl)phenyl]-1-oxobutyl]lysyl-bovine thyroglobulin and (S)-N-[4-[4-[2(Methylamino)propyl]phenyl]-1-oxobutyl]lysyl-bovine thyroglobulin (Fig. 1 structures 3 and 4, respectively) were injected at the rate of 1 mg/mL/month in complete Freund's adjuvant at multiple sites across the animals' backs [*11*]. Selection of bleeds to form the separate amphetamine and methamphetamine antibody pools were based on specific antibody titers and cross-reactivity results obtained in other immunoassays. Antibody titering was performed using the RIA procedure described as follows. Separate standards consisting of 0, 250, 500, and 1000 ng/mL of D-amphetamine and also of D-methamphetamine in pooled urine were assayed with varying amounts of both the antiamphetamine serum and the antimethamphetamine serum diluted in antibody diluent. That combination of dilutions that produced the most nearly equivalent dose response for the two drugs was selected.

RIA Procedure

The assay was performed by pipetting into glass culture tubes 25 μL of urine sample or standard, 200 μL of the combined ^{125}I-amphetamine and ^{125}I-methamphetamine tracer



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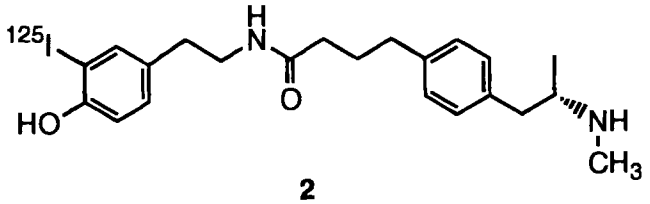

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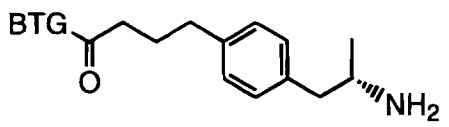

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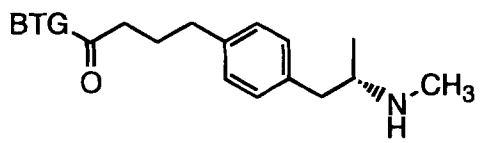

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FIG. 1—*Structures of the radiolabels used in the RIA. (S)-4-(2-Aminopropyl)-N-[2-(4-hydroxyphenyl)-ethyl] benzenebutanamide* (1). *(S)-N-[2-(4-hydroxyphenyl)-ethyl]-4-[2-(methylamino)propyl] benzenebutanamide* (2). *Structures of the immunogens used in the RIA. (S)-N-[4-[4-(2-Aminopropyl)phenyl]-1-oxobutyl]lysyl-bovine thyroglobulin* (3). *(S)-N-[4-[4-[2(Methylamino)propyl]phenyl]-1-oxobutyl]-lysyl-bovine thyroglobulin* (4).

mixture, 500 μL of 2nd antibody reagent, 50 μL sodium periodate reagent, and 200 μL of the combined amphetamine and methamphetamine antibody reagent solution. This mixture was vortexed, incubated for 60 minutes at room temperature, centrifuged at 1500 × g for 10 min, and the supernatants decanted. The pellet was then counted in a gamma counter to obtain cpm values from which a dose response curve could be established.

Results

Figure 1 in Materials and Methods shows the structures of the radiolabeled amphetamine and methamphetamine derivatives and of the immunogens which were used in this assay. All structures were synthesized solely as D-isomers in order to enhance selectivity for the illegal form of the drugs.

Figure 2 illustrates the large dynamic dose response curves that can be achieved with either amphetamine and methamphetamine standards. There are greater than 70,000 dpm's from the zero standard to the 500 ng/mL cutoff calibrator and an additional 20,000 dpm's to the highest level calibrator for an overall dynamic range of greater than 90,000 dpm's. (All data in this figure have been converted to dpm's to standardize the differences seen in gamma counter efficiencies.) At each calibrator level, there is less than a 1.3% difference in dpm's between the response obtained with amphetamine calibrators and that obtained with methamphetamine calibrators. This equivalent response allows either D-amphetamine or D-methamphetamine to be used as calibrators and should result in the equal quantitation of both amphetamine and methamphetamine. To determine that indeed this was the case, spike and recovery studies were conducted: Recovery of D-amphetamine and D-metham-

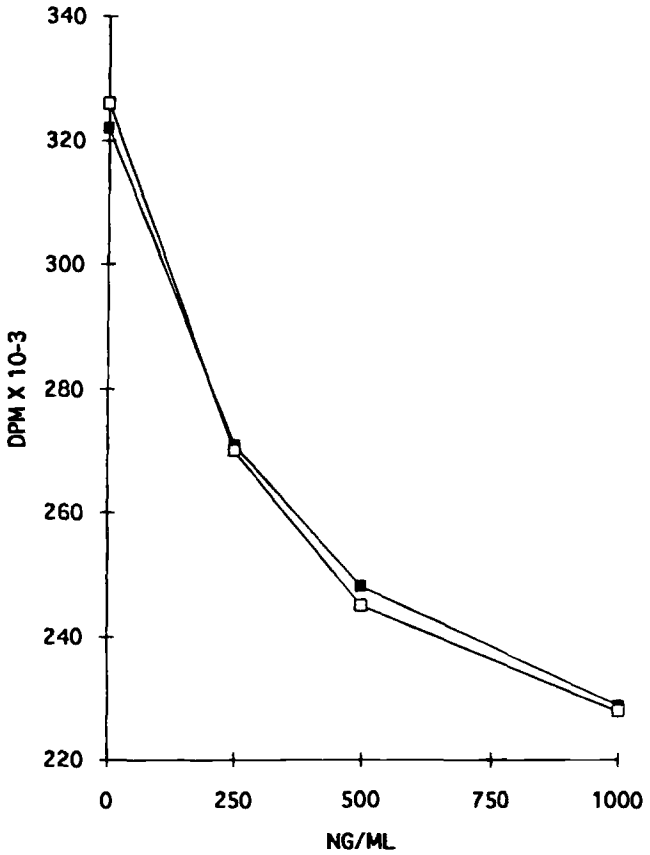

FIG. 2—*Typical dose response curves for amphetamine and methamphetamine. Equivalent dose response curves are demonstrated for both amphetamines. Recovery of either drug can be obtained using only D-amphetamine as calibrators. Legend:* ■ *amphetamine,* ❑ *methamphetamine.*

phetamine in the RIA was evaluated by assaying controls spiked with 250 to 600 ng/mL of the two drugs and determining the ng equivalents/mL obtained by reading off a D-amphetamine standard curve. These results are shown in Table 1. It can be observed that the results obtained for all drug spikes are within 10% of their theoretical value. Additionally, the combined recovery of amphetamine and methamphetamine was also assessed. Control urine was spiked with 25 to 500 ng/mL of both drugs and assayed. Table 2 shows the results that were obtained. It can be seen that when both drugs are present, the determined ng equivalents/mL exceeds the total amount of drugs actually spiked into the sample.

The analytical and clinical sensitivity of this assay were also studied. Analytical sensitivity was determined by assaying diluted aliquots of a 250 ng/mL D-amphetamine control and of the 0 ng/mL control in pooled human urine in replicates of twelve. The lowest concentration of amphetamine tested which could be distinguished from the 0 ng/mL control with 95% confidence was 25 ng/mL. Clinical sensitivity was determined by testing 88 urine samples that had been prescreened with a commercially available enzyme immunoassay and found to be negative. The mean value for the samples was 21 ± 41 ng/mL with a range of −88 to 173 ng/mL. With a criterion of mean ± 2 SD, the clinical sensitivity of the assay was therefore 202 ng/mL.

A key area of concern in amphetamines drug of abuse screening is the cross-reactivity of the immunoassay to β-hydroxylamines. To evaluate this assay's response to these com-

TABLE 1—*Recovery study—individual drugs.*

Drug	Spiked conc. (ng/mL)	Mean conc. found (ng/mL)	Recovery (%)
Amphetamine	250	271	108
	400	414	104
	500	551	110
	600	607	101
Methamphetamine	250	259	104
	400	415	104
	500	550	110
	600	634	106

NOTE—Table 1 demonstrates equal recovery of both D-amphetamine and D-methamphetamine in spiked urine samples when measured against D-amphetamine calibrators.

TABLE 2—*Recovery study—combined drugs.*

Spiked methamphetamine concentration (ng/mL)	Spiked amphetamine concentration (ng/mL)					
	25	50	100	200	250	500
	Concentration found (ng/mL)					
25	62	84	194	412	446	773
50	72	183	303	461	763	961
100	221	280	434	677	896	1824
200	305	450	552	1133	1237	2091
250	441	509	899	1450	1652	2540
500	582	1300	1617	1710	2373	3446

NOTE: Table 2 illustrates the enhanced nonlinear response observed when both amphetamine and methamphetamine are present in the sample.

pounds, the common OTC β-hydroxylamines were tested at 1 mg/mL in urine both with and without the addition of periodate reagent. When periodate was used, as described in the RIA Procedure, the final concentration in the reaction mixture was 5.1 mM. In the assays without periodate reagent, 50 μL of distilled water was substituted for this reagent into the reaction mixture of both standards and unknowns. The results, shown in Fig. 3, indicate that without periodate, (+)pseudoephedrine is the most reactive of the β-hydroxylamines tested, with approximately 0.2% cross-reactivity. With periodate, however, this cross-reactivity is completely eliminated. The most cross-reactive of the compounds tested in the presence of periodate is (−)ephedrine. However, its level of cross-reactivity was reduced from 0.2% without periodate to only 0.01% with the oxidizing reagent. Addition of periodate into this assay, we conclude, is essential in order to reduce or eliminate interferences from the β-hydroxylamines. To further evaluate the specificity of the assay, other structurally related compounds which are not β-hydroxylamines, including the non-controlled L-isomers of the two drugs, were also tested. These results are shown in Table 3.

The actual clinical performance of the RIA was challenged by a panel of GC/MS NIDA confirmed positives and by a panel of samples that were shown to be GC/MS NIDA negative. One hundred and eleven samples that had screened positive in a clinical laboratory and subsequently confirmed by GC-MS to contain either ≥500 ng/mL amphetamine or ≥500 ng/mL methamphetamine plus 200 ng/mL amphetamine (current NIDA guideline for confirmation of amphetamines) were tested in the RIA. All 111 samples were identified as positive. Additionally, 60 samples which, with other immunoassays had produced significantly elevated or positive results, were also tested in the RIA. These 60 samples were subsequently shown by GC/MS to be negative for both amphetamine and methamphetamine.

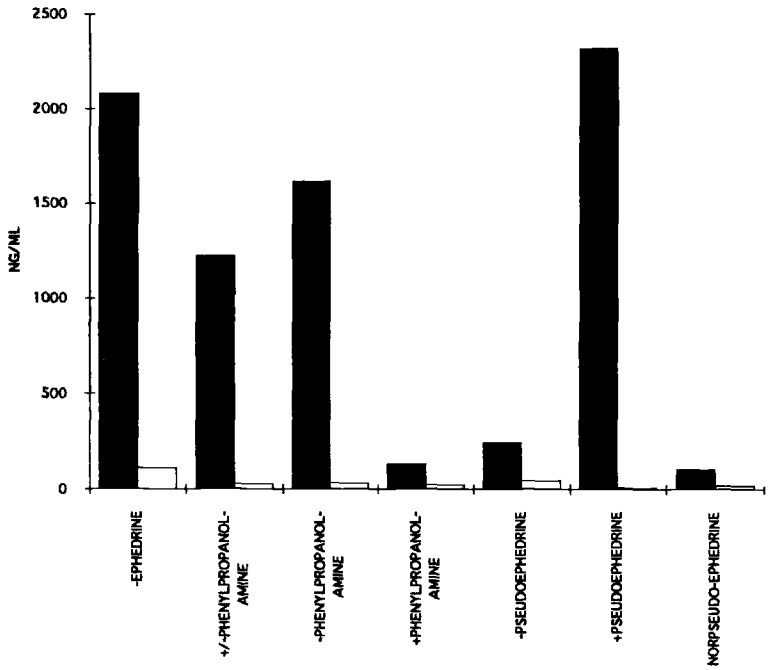

FIG. 3—*Simultaneous periodate treatment of β-hydroxylamines in the RIA. The effect of sodium periodate oxidation on β-hydroxylamines was tested with compounds spiked at 1 mg/mL in urine. The results are expressed as ng amphetamine equivalents/mL. Legend:* ■ *without periodate reagant,* ❑ *with periodate reagent.*

TABLE 3—*Cross-reactivity to structurally related compounds.*

Compounds tested	Concentration tested (ng/mL)		
	1000	10,000	100,000
	Concentration found (ng equiv./mL)		
Phentermine	39	164	>1000
Tyramine	0	71	795
MDA[a]	>1000	>1000	>1000
MDMA[b]	799	>1000	>1000
B-Phenethylamine	0	153	>1000
L-Amphetamine	—	462	>1000
L-Methamphetamine	—	239	>1000
p-Hydroxyamphetamine	315	>1000	>1000
D,L-Amphetamine	665	—	—
D,L-Methamphetamine	623	>1000	>1000
Propylhexidrine	121	703	>1000

NOTE: Table 3 illustrates the low cross-reactivity of the RIA to structurally related compounds that are not β-hydroxylamines. Compounds were tested at three concentrations.

[a]Methylenedioxyamphetamine.

[b]Methylenedioxymethamphetamine.

Fifty-six of the samples were also negative in the RIA. Based on those 171 samples tested, as shown in Fig. 4, qualitative correlation of the RIA with GC/MS was determined to be 98%. Three of the four GC/MS discrepant samples contained detectable levels of amphetamine and methamphetamine, but were below the established GC-MS NIDA cut-off. Results obtained for these four samples are shown in Table 4. In another study, the ability of the periodate reagent to eliminate false-positive results caused by β-hydroxylamines was challenged further by testing clinical samples with and without periodate treatment as described for cross-reactivity testing. Nineteen GC/MS negative samples that had exceeded a 1000 ng/

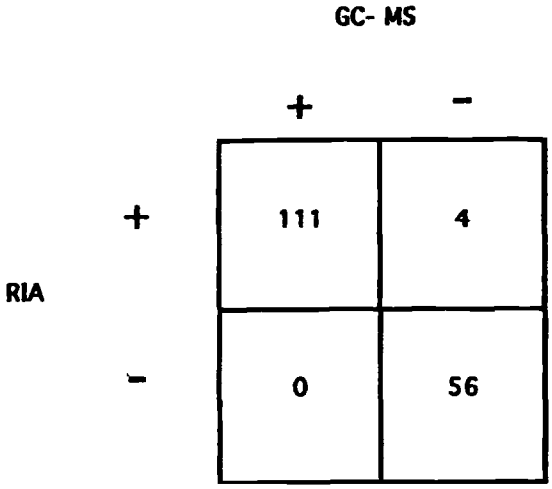

FIG. 4—*Clinical correlation of the RIA with GC/MS. Qualitative correlation of the RIA with GC/MS is shown. From 171 clinical samples tested, a 98% agreement between the two methods was observed.*

TABLE 4—*GC/MS and RIA discrepant samples.*

Sample	GC/MS (ng/mL)		RIA (ng equiv./mL)
	Amp.	Meth.	Amp. and Meth.
1	141	>3000	>1000
2	243	432	>1000
3	85	217	>1000
4	0	0	583

NOTE: Table 4 shows the GC/MS and RIA values obtained for the four discrepant of 60 samples that were elevated or positive in other immunoassays and that were shown by GC/MS to be negative for amphetamines.

mL cut-off level for amphetamine or methamphetamine in at least one commercially available immunoassay were tested. Even with a 500 ng/mL cut-off, only seven of these samples were positive in the RIA without periodate, while with periodate, none were positive. Quantitative results obtained for these seven samples are shown in Table 5.

Discussion

The concept of dual antigen detection in an immunoassay is not new [*12,13*] but is particularly appropriate for the amphetamines since both amphetamine and methamphetamine are pharmacologically active, are related metabolically, are both abusable drugs, and can be illegally synthesized from similar starting materials. Presented here is a new RIA for the detection of either amphetamine or methamphetamine with an equal dose response, or the detection of both drugs simultaneously, while maintaining low cross-reactivity to β-hydroxylamines. Dose response curves for D-amphetamine and D-methamphetamine are essentially superimposable, permitting the use of D-amphetamine as calibrators. When this assay is detecting a mixture of both drugs simultaneously an enhanced non-linear response is observed. This effect is a result of the fact that the RIA measures a combined signal (gamma emission) from two independent and nonlinear reactions. This response has been observed both with spiked urine samples (Table 2) and with clinical specimens. As is seen in Table 4 three samples (1 to 3) contained GC/MS detectable levels of both amphetamine and methamphetamine but were GC/MS negative according to present NIDA guidelines. Although these samples contain drug, they must be viewed as administrative false positives. Based on our clinical database, however, we believe this type of sample is a relatively low percentage of the total population of samples tested for amphetamines.

TABLE 5—*Samples with and without periodate reagent in the RIA.*

Sample	GC/MS (ng/mL)		RIA (ng equiv./mL)	
	Amp.	Meth.	−Periodate	+Periodate
1	0	0	>1000	84
2	0	0	>1000	80
3	0	0	784	181
4	0	0	514	210
5	0	0	>1000	106
6	0	0	>1000	247
7	0	0	589	276

NOTE: Table 5 shows the effect of periodate treatment on clinical samples containing β-hydroxylamines.

A more critical issue that must be addressed in amphetamines screening assays is the problem associated with false-positive results arising from the presence of β-hydroxylamines or from the legal isomeric forms, L-amphetamine and L-methamphetamine. This RIA has reduced β-hydroxylamines cross-reactivity through the incorporation of periodate oxidation during the assay incubation time and has also substantially reduced cross-reactivity to the legal isomers by using antibodies and radiolabel both generated from the illegal isomers, D-amphetamine and D-methamphetamine. These claims are supported in the data presented in Tables 3 and 5. Between 10 000 and 100 000 ng/mL of L-amphetamine or L-methamphetamine are needed to produce a positive result. In addition, all except one sample tested in the RIA that were elevated or screened positive in other commercial assays but contained absolutely no amphetamines, were negative in the RIA (Tables 4 and 5). The one sample that also screened positive in the RIA (Table 4) is known from additional GC/MS data, not to contain any of the common β-hydroxylamines. The exact cause of this false positive result is not known at this time. As is demonstrated in Fig. 3 the RIA is capable of eliminating very high levels of the β-hydroxylamines. These levels are consistent with the concentrations of these compounds that may appear in urine with the use of OTC medications for colds or diets [*14*].

The new RIA amphetamines assay was developed for reasons discussed and also in anticipation of a desire or need for a 500 ng/mL cutoff for the detection of amphetamines. This assay affords the opportunity of screening at a lower cutoff without increasing the already difficult problem of false-positives seen from β-hydroxylamines. Even with the lower cutoff this assay presents no serious problem with either the β-hydroxylamines or the legal isomers of the amphetamines.

In conclusion, we have presented a new radioimmunoassay for the detection of amphetamine and/or methamphetamine which is specific for the illegal D-form of the drugs or for their designer-drug counterparts, MDA and MDMA, while simultaneously maintaining low cross-reactivity to other related substances through the use of specific antibodies and of periodate oxidation of β-hydroxylamines.

Acknowledgment

The authors gratefully acknowledge the excellent technical assistance of Ms. Uwayemen Akhabue and also of Dr. Ken Savoca.

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