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A biotin–avidin based screening test for methamphetamine in urine*

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Abstract: A biotin–avidin based screening test for methamphetamine in urine samples has been developed. The assay method utilizes the immunoprecipitin reaction between an antibody to methamphetamine and a conjugate prepared by complexing avidin with a biotinyl amphetamine derivative. The rate of the immunoprecipitin reaction is monitored on the Beckman ARRAY® 360 nephelometer. Methamphetamine inhibits the precipitin reaction, and the extent of inhibition allows the quantitation of methamphetamine in the urine samples. Using a cut-off value of 0.7 $\mu g\ ml^{-1}$, the assay correctly predicted 83 of 84 samples (98.8%) confirmed to be positive by GC–MS (>500 ng ml^{-1}). Of 59 GC–MS confirmed negative samples, 46 samples were found to be negative by this method as compared to 34 samples determined with the EMIT® assay. Within-run and between-run relative standard deviations near the cut-off value were less than 4%. Cross-reactivity with amphetamine was <7%.

Keywords: *Methamphetamine; immunoprecipitin reaction; biotin–avidin.*

Introduction

Methamphetamine was synthesized as a pharmacological agent in 1919. Many of the pharmacological effects of methamphetamine, including bronchodilator, respiratory stimulant, and analeptic properties, were noted by Alles [1] in 1933. Methamphetamine is a potent sympathometric amine with rapid and powerful central nervous stimulation.

The continued concern regarding methamphetamine use and abuse necessitates the laboratory to maintain the capability of testing for this drug in biological fluids. The specific methods chosen by a laboratory will depend on a number of factors, including cost, workload, turnaround time, sensitivity required, and reliability. The most popular analytical methods are immunoassays for screening and gas chromatography–mass spectrometry (GC–MS) or liquid chromatography–mass spectrometry (LC–MS) for confirmatory tests [2]. GC–MS is generally considered to be the most conclusive method of confirming the presence of a drug in urine. GC–MS combines the efficient separating power of gas chromatography with the high sensitivity and specificity of mass spectrometric detection. Among the commercial immunoassay methods for assaying methamphetamine are the Syva EMIT® [3, 4], the Roche Abuse-screen® ONLINE™ [5], the Roche Abuse-screen® RIA, and the Abbott TDX® [3, 6, 7]. The biotin–avidin nephelometric procedure described in this paper represents a new immunoassay method for the specific measurement of methamphetamine in urine. The assay utilizes the biotin–avidin immunoprecipitin procedure previously described by Oh and Sternberg [8] for therapeutic drugs in serum samples. The principle of the biotin–avidin assay as applied to methamphetamine is shown in Fig. 1. The method is sensitive, specific, rapid and reliable.

Materials and Methods

Chemicals and biochemical materials

The chemical and biochemical materials used in this study were obtained from the following sources: biotin from Schweizerhall (Piscataway, NJ, USA); *N*-hydroxy-succinimide (NHS), dicyclohexylcarbodiimide (DCC), *N*,*N*-carbonyldiimidazole (CDI), 1,6-hexanediamine, 6-aminocaproic acid, aluminium chloride, trifluoracetic anhydride, dimethylformamide (DMF), hydrogen bromide as a 30% w/w solution in acetic acid, dimethylaminocinnamaldehyde spray reagent, Amberite IRA-400(OH) ion-exchange resin, and Dowex 50X2-200 ion-exchange resin from

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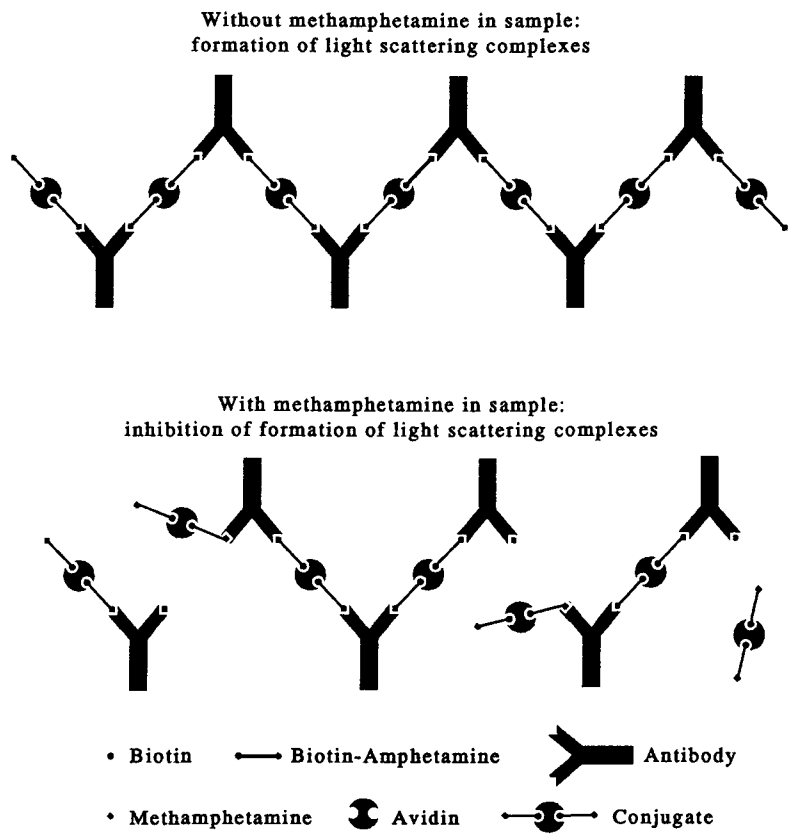

Figure 1
Biotin–avidin based nephelometric assay of methamphetamine.

Aldrich (Milwaukee, WI, USA); *d*-methamphetamine sulphate and succinic anhydride from Sigma (St Louis, MO, USA); *d,l*-methamphetamine, *d,l*-amphetamine and *d,l*-methylenedioxyamphetamine (MDA) from Radian (Austin, TX, USA); cross-reactants with the exception of *d,l*-amphetamine and MDA from Alletch-Applied Science (State College, PA, USA); rabbit anti-methamphetamine serum from Chem-Scan (La Handa, CA, USA); bovine serum albumin (fraction V) from Pentex (Kankakee, IL, USA), avidin from Boehringer (Mannheim, Germany); urine specimens with assigned GC–MS values from Damon Laboratories (Rancho Cucamonga, CA, USA).

Buffers

Buffer A. Phosphate-buffered saline (PBS) solution containing 0.01 M NaH_2PO_4–Na_2HPO_4, 0.15 M NaCl and 0.1% sodium azide, pH 7.0.

Buffer B. Citrate-buffered saline (CBS) solution containing 0.025 M sodium citrate, 5 mM EDTA, 0.15 M NaCl and 0.1% sodium azide, pH 6.0.

Buffer C. Phosphate buffer containing 0.1 M NaH_2PO_4–Na_2HPO_4, 0.15 M NaCl, 0.1% sodium azide and 4.4% polyethylene glycol, pH 7.0.

Biotinyl amphetamine (V)

The preparation of the biotinyl amphetamine (V) is outlined in Fig. 2.

*4-Succinyl-*n*-trifluoracetylamphetamine (III).* To a solution of *d*-amphetamine (1.2 g) in anhydrous benzene (20 ml) at 0°C was added trifluoroacetic anhydride (2.08 g), and the resulting mixture was stirred at room temperature for 1 h and refluxed for 3 h. After removal of the solvent, recrystallization of the residue from acetone–ether yielded 1.96 g of *N*-trifluoracetylamphetamine (II, m.p. 79–81°C). 4-Succinyl-*N*-trifluoracetylamphetamine (III) was prepared by refluxing a solution of II (0.46 g), succinic anhydride (0.2 g) and aluminium chloride (0.6 g) in dichloro-

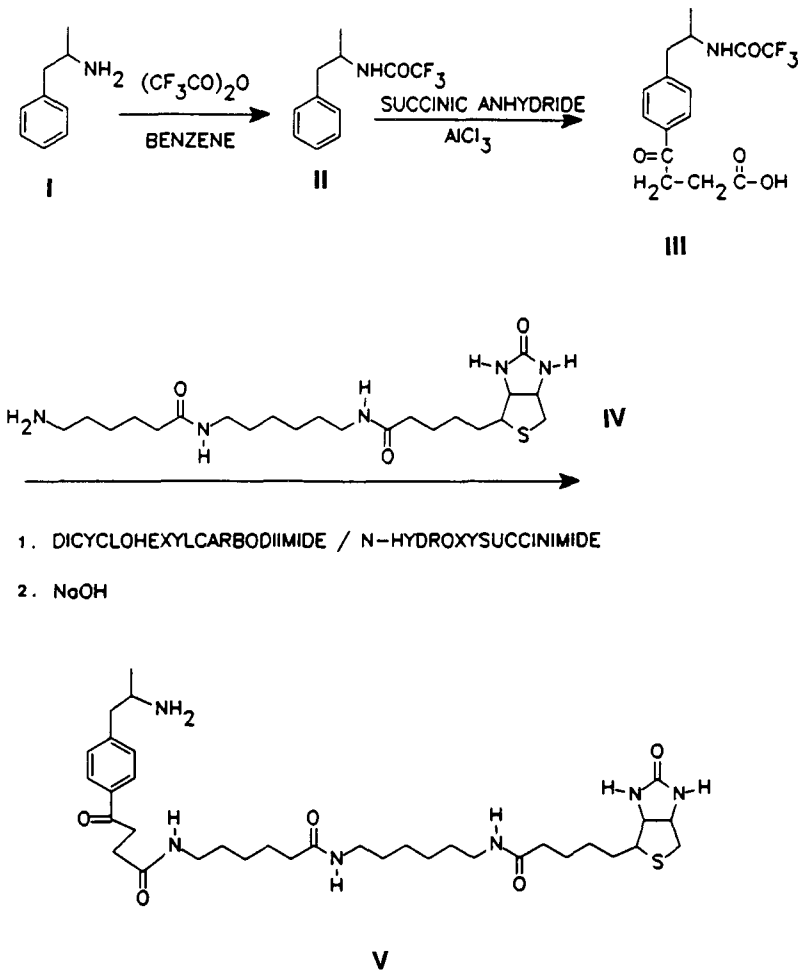

Figure 2
Preparation of biotinyl amphetamine label (V).

methane (20 ml) for 15 h. After addition of 5.2 ml of cold hydrochloric acid (0.04 N), III was collected by filtration, washed with 5% sodium bicarbonate solution followed by water, and recrystallized from ethyl acetate–ether (yield 0.1 g, m.p. 168–169°C).

N-*(Aminocaproyl)*-N′-*biotinyl hexylenediamine (IV).* A solution of 1,6-hexanediamine (4.64 g) in dry DMF (100 ml) was added dropwise with stirring to a solution of biotin *N*-hydroxysuccinimide ester (5 g), which was prepared from biotin and NHS by the method of Bayer [9]. After overnight stirring, the solvent was removed to dryness, and the resulting residue was purified on a silica gel column. Elution with methanol and then methanol–chloroform–ammonium hydroxide (9.5:9.5:1, v/v/v) afforded the pure biotin–hexanediamine (1.2 g).

To a solution of carbobenzoxy-ε-aminocaproic acid [10] (0.77 g) in dry DMF (30 ml) at 75°C was added carbonyldiimidazole (0.535 g), and the reaction mixture was stirred at this temperature for 30 min and at room temperature for 2 h. *N*-hydroxysuccinimide (0.38 g) was introduced followed by overnight stirring, introduction of the biotin–hexanediamine (1 g) in DMF (100 ml), and an additional stirring for 15 h. The solvent was removed, and the residue was triturated with 6 N HCl, filtered, and recrystallized from methanol to give 1.5 g of white solid. The white solid was then suspended with stirring in 3 ml of a 30% w/w solution of hydrogen bromide in acetic acid for 5 h, after which water (10 ml) and methanol (20 ml) were added. The resulting solution was neutralized with Amberlite IRA-400(OH) ion-exchange resin, and filtered. After the filtrate was evaporated to dryness,

the residue was recrystallized from methanol to give 1 g of IV. The product was TLC pure, positive to ninhydrin spray for the presence of amino group, and positive to dimethylaminocinnamaldehyde spray for the presence of biotin.

Biotinyl amphetamine (V). A solution of III (0.06 g), dicyclohexylcarbodiimide (0.011 g) and NHS (0.021 g) in DMF (5 ml) was stirred at room temperature for 1 h, and IV (0.083 g) was added. After 2 days of stirring and evaporation of the solvent, the reaction product (V, 30 mg) was isolated by preparative TLC using 20% methanol in chloroform as the developing solvent. The trifluoroacetyl group in the reaction product was then removed by hydrolysis in 1 ml of 2 N sodium hydroxide–methanol (4:5, v/v) for 2 h, and the resulting solution was neutralized with Dowex 50X2-200 ion-exchange resin. The solvent was evaporated, and the residue was purified by preparative TLC employing 50% methanol in chloroform as the eluent to give 16 mg of V.

Avidin–biotinyl amphetamine conjugate

The avidin–biotinyl amphetamine conjugate was prepared by mixing a solution of avidin (44 mg) in 0.1 M phosphate buffer (pH 7.4, 4 ml) and a solution of V (7 mg) in methanol (1 ml). After 10 min of reaction followed by dialysis with the citrate buffered saline (CBS, buffer B), the conjugate concentrate was diluted with CBS to a working concentration of 0.2 mg ml^{-1} for immunoassays.

d,l-*Methamphetamine standards*

d,l-Methamphetamine was obtained from Radian as a methanol solution containing 1 mg ml^{-1} of the drug. The methanol stock solution was diluted in the Bio-Rad drug negative urine control to give the *d,l*-methamphetamine assay standards consisting of six levels at 0.0, 0.3, 0.5, 1.0, 2.0 and 3.0 μg ml^{-1}.

Antibody reagent

Rabbit anti-methamphetamine serum was obtained from Chem-Scan, and was diluted in phosphate buffered saline (buffer A) containing 0.2% BSA for assays.

Assay protocol on ARRAY® 360

Reagents and buffers for the biotin–avidin based methamphetamine assay were introduced and mixed automatically by the ARRAY® 360 nephelometer according to the following protocol and addition sequence: (1) introducion of reaction buffer (buffer C, 600 μl), (2) introduction of sample (42 μl, 1:6 on-line dilution in buffer C), (3) addition of avidin–biotin amphetamine conjugate (42 μl), (4) addition of antibody (42 μl) to trigger reaction, and (5) measurement of nephelometric response.

EMIT® reagents and assay on Cobas Bio®

The EMIT® d.a.u. MAB amphetamine assay kit (Syva) was employed in the method comparison study. The reagents were reconstituted according to the manufacturer's instructions and stored at 4°C until use. The assays were adopted for the Cobas Bio® centrifugal analyser according to protocols supplied by the kit manufacturer.

Results

Methamphetamine standard curve

A typical standard curve for the biotin–avidin urine methamphetamine assay on the ARRAY® 360 is shown in Fig. 3. Each point on the curve is the mean of two determinations. The assay covered a measuring range of 0–3 μg ml^{-1}, which corresponded to nephelometric response of 1600–480 rate units, respectively. The assay, which has a detection limit of ≈0.1 μg ml^{-1}, utilized a cut-off concentration of 0.7 μg ml^{-1} or rate unit of 1115 for identifying methamphetamine positive samples. The standard curve was steep, and its dose response compared favourably with that obtained with the EMIT® reagents on the Cobas Bio® from which the rate of change in absorbance was only 0.117 absorbance units (at 340 nm) per minute over the same concen-

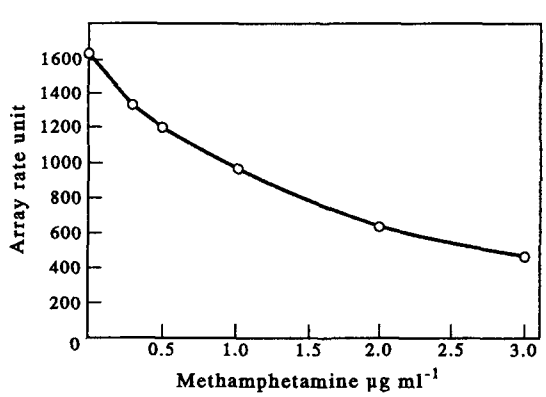

Figure 3
Methamphetamine standard curve.

tration range. To assess the reproducibility of the biotin–avidin based methamphetamine standard curve, nine runs were monitored over a period of 6 weeks. It was found that the rate unit at each concentration level varied by no more than 4% for the time period chosen.

Assay precision

Within-run and between-run precisions were evaluated on two urine samples containing 0.8 and 1.7 μg ml^{-1} methamphetamine. The within-run precision for each of the two samples was determined in 20 replicates. For between-run precision, 16 replicates for each sample were obtained from eight assays covering a period of 1 month, with each assay run in duplicate. The precision results are shown in Table 1, which indicated RSDs of less than 4% at near and above the cut-off concentration levels. Because both the within- and between-run RSDs obtained were less than 4%, we consider the precision acceptable for the methamphetamine assay.

Table 1
Precision studies for the analysis of methampetamine in urine

		Within-run precision		
Sample	*n*	Mean (μg ml^{-1})	SD (μg ml^{-1})	RSD (%)
Low	20	0.80	0.024	2.89
High	20	1.68	0.033	1.89
		Between-run precision		
Sample	*n*	Mean (μg ml^{-1})	SD (μg ml^{-1})	RSD (%)
Low	16	0.81	0.029	3.56
High	16	1.69	0.058	3.45

Specificity

The cross-reactivity of the methamphetamine metabolites and structurally related compounds was studied, and is shown in Table 2. The cross-reactivity of a cross-reactant at a given concentration was assessed by running the sample as unknown in the methamphetamine assay. Cross-reactivity is defined as the

Table 2
Results of the cross-reactivity study

Cross-reactant	Cross-reactant conc. used in study (x μg ml^{-1})	Observed equivalent *d,l*-methamphetamine conc. (y μg ml^{-1})	% Cross-reactivity = 100 × (y)/(x)
d,l-Methamphetamine	—	—	(100.0)
d-Methamphetamine	1	1.12	112.0
	2	2.20	110.0
l-Methamphetamine	1	0.77	77.0
	2	1.39	69.5
	5	2.56	51.2
d,l-Amphetamine	1	0.06	6.0
	2	0.12	6.0
	5	0.29	5.8
	10	0.52	5.2
d-Amphetamine	5	0.23	4.6
	10	0.54	5.4
	50	2.16	4.3
l-Amphetamine	5	0.25	5.0
	10	0.45	4.5
	50	1.19	2.4
	100	2.06	2.1
Methylene dioxyamphetamine	5	0.46	9.2
(MDA)	10	0.90	9.0
Codeine	100	0.16	0.2
Oxazepam	100	0.00	0.0
Secobarbital	100	0.00	0.0
d,l-Methadone	100	0.01	0.0
Phencyclidine	100	0.01	0.0
Propylamphetamine	10	0.65	6.5
	100	1.77	1.8
Fenfluramine	10	1.36	13.6
	100	2.78	2.8
Phenmetrazine	10	0.60	6.0
	100	1.96	2.0
Methaqualine	100	0.00	0.0
l-Ephedrine	1	0.30	30.0
	10	1.33	13.3

apparent *d,l*-methamphetamine concentration observed due to the presence of the cross-reactant, divided by the concentration of the cross-reactant in the sample.

As can be seen from Table 2, the biotin–avidin methamphetamine assay developed here measures predominantly methamphetamine with cross-reactivity to amphetamine of no more than 7%. L-ephedrine at 1 μg ml^{-1} cross-reacted by as much as 30% with the antibody selected for this assay.

Correlation study

A total of 143 urine specimens consisting of 84 GC–MS positive and 59 GC–MS negative samples were obtained from Damon Laboratory. The samples were pre-analysed by GC–MS by Damon Laboratory and determined as positive or negative using the NIDA cut-off of 0.5 μg ml^{-1} for methamphetamine. These samples were evaluated on the ARRAY® 360 in duplicate in five separate runs using the biotin–avidin nephelometric method, and also with the EMIT® reagent on the Cobas-Bio in duplicate in 14 separate assays.

Of the 143 specimens, 107 samples had GC–MS methamphetamine values of ≤3 μg ml^{-1}, and the GC–MS results of these samples were compared to those assayed with the biotin–avidin and EMIT® methods (Figs 4 and 5). The biotin–avidin method gave one false negative and 13 false positive samples, while the EMIT® assay resulted in no false negative but 25 false positive answers. Additionally, neither method gave any false negative value for the 36 remaining GC–MS positive samples (>3 μg ml^{-1}).

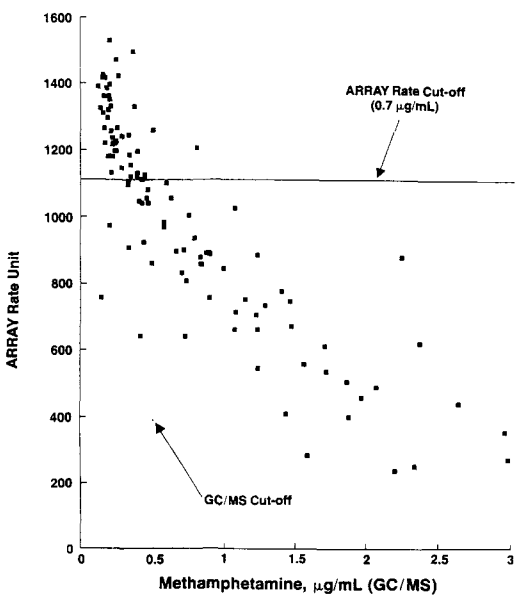

Figure 4
ARRAY vs GC–MS (n = 107).

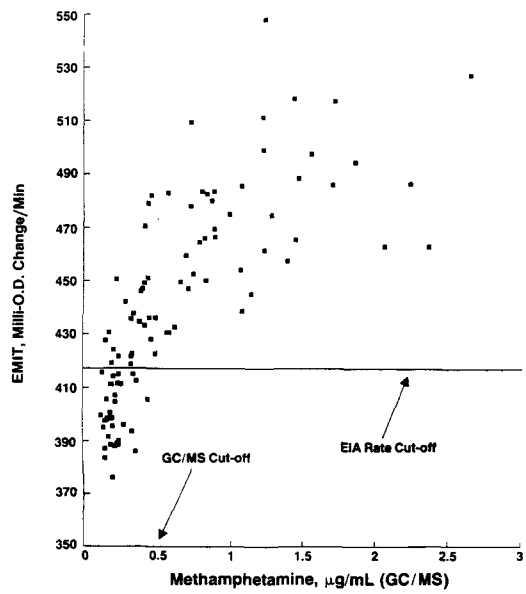

Figure 5
EMIT vs GC–MS (n = 107).

The correlation results for all 143 samples are summarized in Table 3, which shows the same number of positive, negative, false positive and false negative samples as described above. With the biotin–avidin method, the predictivity for positive and negative samples were 99 and 78%, respectively. With the EMIT® reagents on the same samples, the predictivity for negative samples was only 58%, and all positive samples became positive. It is clear from these data that the biotin–avidin reagents developed for the ARRAY® 360 compared favourably with the EMIT® reagents in the screening of urine methamphetamine samples.

Table 3
Analysis of the correlation study

		GC–MS	
n = 143		+	–
Array	+	83	13
(Cut-off: 0.7 μg ml^{-1})	–	1	46
EMIT	+	84	25
(Cut-off: 1.0 μg ml^{-1})	–	0	34

Discussion

The purpose of this study was to develop a simple, rapid and reliable method to assay drugs of abuse in urine specimens. The biotin–avidin nephelometric procedure described in this report has satisfied these criteria. The assay is homogeneous, and involves just the mixing of reagent components, i.e. the antibody, the avidin–biotin drug conjugate and the standards, in a reaction buffer. The immunoprecipitin reaction is fast, and completed in less than 2 min. The rate of formation of immunocomplex from the antibody and the conjugate can be monitored on the ARRAY® 360 nephelometer, and is inversely proportional to the amount of methamphetamine in the sample. The biotin–avidin nephelometric method has an advantage for urine over serum samples as the former matrix results in much lower initial background light scattering, and therefore, more urine sample can be utilized in the assays.

The biotin–avidin methamphetamine assay performed well in areas of precision, reproducibility, and predictivity for drug positive samples as compared to the EMIT® assay. The standard curve was reproducible over a period of 9 weeks. The reproducibility coupled with the steepness of the dose–response curve resulted in precision of <4% at the 0.8 and 1.44 $\mu g\ ml^{-1}$ concentration levels. The assay has predictive values of 99 and 78%, respectively, for identifying methamphetamine positive and negative samples using the cut-off value of 0.7 $\mu g\ ml^{-1}$. The predictive values compared favourably with the respective values of 100 and 58% obtained by EMIT®. It is evident from these data that the biotin–avidin nephelometric method predicted fewer false positives than EMIT® (22 vs 42%), and therefore fewer confirmatory tests by GC–MS, which is time consuming and expensive, would be required with the former method. With the antibody selected, the assay measured mainly methamphetamine with ≈10% cross-reactivity to amphetamine. L-ephedrine interfered with the assay.

The biotinyl amphetamine–avidin conjugate was originally intended for the amphetamine assay, which is under development. We were somewhat surprised by the results obtained with the amphetamine conjugate paired with the methamphetamine antibody from Chem-Scan, as it exhibited not only good immunoreactivity and steep dose–response to methamphetamine, but also low cross-reactivity (<7%) to amphetamine.

The formulation of the biotin–avidin conjugate component is easy to perform, and reproducible. The procedure involves merely the mixing of avidin and a biotinyl drug derivative followed by removal of the excess organic components by dialysis. The conjugate is stable, with >85% retention of immunoreactivity after 7 days of heat stressing at 45°C.

It is concluded that the biotin–avidin nephelometric procedure provides a convenient and simple method for detecting methamphetamine in urine samples.

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