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Studies on Fluorescamine: Part I—Applications of Fluorescamine in Forensic Toxicological Analysis

Weigele and co-workers [*1,2*] reported the structure and synthesis of the reagent 4-phenylspiro [furan-2(3H), 1'-phthalan]-3,3'-dione (fluorescamine), which reacts with substances containing primary amino groups to yield highly fluorescent products. Recently, Undenfriend and co-workers [*3–5*] published a paper describing the use of fluorescamine in biochemical analysis. This reagent was of interest because of its possible use to improve the detection and differentiation of amphetamine and its relatives in biological samples and solid dosage forms. The main subject of this paper is to present data which clearly demonstrate the use of fluorescamine in forensic toxicological analysis of amphetamine and differentiation from methamphetamine. This reagent also has potential application for analysis of other drugs containing a primary amine group.

Two compounds of primary interest to the law enforcement effort are amphetamine and methamphetamine, although a few other amphetamine-like drugs are also federally controlled. The physiological effects of these two drugs are similar [*6–8*], methamphetamine having a slightly greater stimulant effect on the central nervous system. Amphetamine and methamphetamine are both optically active compounds. The dextro isomers are about twice as active as the recemic mixtures, however, all isomers are under Federal control and are listed in Schedule II of Public Law 91-513.

An excellent review of existing methods for analysis of amphetamine analogs has been published [*9*]. Miles and Schenk [*10*] made use of the natural fluorescence of phenylethylamines to assay these compounds in pharmaceutical preparations. Few attempts have been made to form fluorescent derivatives of amphetamine and related substances.

In this work, fluorescamine is used to form fluorescent derivatives. It has also been found very simple to further analyze the fluorescent derivative formed in the spot test by thin-layer chromatographic analysis.

Methods and Materials

Fluram™ (fluorescamine) was purchased from Roche Diagnostics, Division of Hoffman-La Roche, Inc., Nutley, N.J. The reagent is supplied in vials containing 100-mg fluorescamine crystals. Fluram is stable at room temperature in both solution and powder form; thus, refrigeration is not recommended by the manufacturer.

The fluorescamine is prepared into a working solution by dissolving 50 mg of fluorescamine in 100 ml of acetone. Fluorescamine has minimal solubility in water

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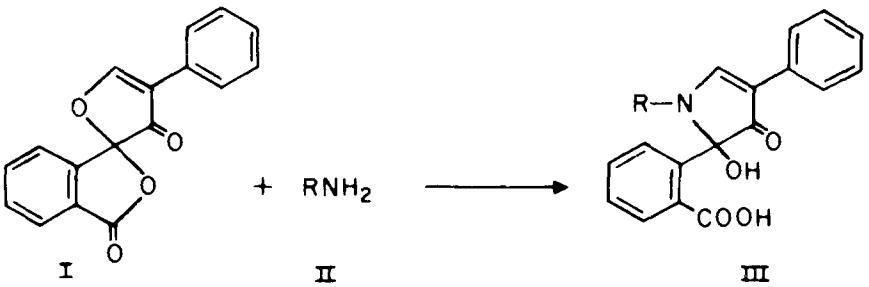
and it will decompose in the presence of water. In the assay, excess reagent is hydrolyzed to water-soluble nonfluorescent products [3].

In Fig. 1, fluorescamine (I) reacts with primary amines (II) to form intensely fluorescent substances (III), providing the basis for a rapid and highly sensitive assay for compounds containing a primary amine group, such as amino acids, primary amines, peptides, and proteins [3]. The reagent (I) does not react with secondary or tertiary amines. Thus, it provides a quick method to distinguish different types of amines; this has been found to be very helpful to distinguish between amphetamine and methamphetamine. Amphetamine is a primary amine and yields an intensely blue-green fluorescent product in the fluorescamine test and methamphetamine does not yield a fluorescent product.



I II III

FIG. 1—*The reaction between Fluram®* (I) *and a primary amine* (II).

The spot test procedure used was to add to a tile-welled spot plate two drops of borate buffer, pH 9.0, and check the fluorescence. All fluorescence examinations were performed in a Chromato-Vue® box (manufactured by Ultra-Violet Products, Inc., San Gabriel, Calif.,) using the long wave (366 nanometer) for excitation. In the spot test the fluorescence is so strong that it can be detected in room light with a small hand ultraviolet (UV) source for excitation. Then a small amount of the exhibit sample was added to the borate buffer and the fluorescence was determined. It is important to check the cleanliness of the plate and the natural native fluorescence of the substance for proper interpretation of the results. The test was completed by adding one drop of the fluorescamine acetone solution and checking the long-wave fluorescence. A positive test was the formation of a very intense blue-green fluorescent product. A negative test was one that did not form a fluorescent product or had fluorescence of another color due to the native fluorescence of the substance.

Quite often it was found to be helpful to analyze the intensely blue-green product further; this was done by thin-layer chromatography (TLC). Two to three microlitres of the spot test were applied to a silica gel thin-layer chromatogram which did not contain any fluorescent material. Standard fluorescent products were prepared from known substances and also applied to the chromatogram. The chromatograph was then developed in a chloroform:methanol system (90:10). After development the fluorescent products were located with the aid of long-wave UV light and the R_f values compared to that of standard compounds. Since very small amounts of the aqueous reaction mixture were required for thin-layer chromatography analysis, there were no problems with drying of the chromatogram before chromatography.

Amphetamine present in urine specimens (10 ml) was isolated by extraction at pH 11 with two volumes of chloroform:isopropanol (3:1) [11]. The solvent extracts were treated with 0.10 ml of acidified methanol (0.1 mol sulfuric acid per litre of methanol) and evaporated. The residue was redissolved in 50 μl of methanol for application to a thin-layer plate.

Simultaneously, in duplicate, drug-free urine and amphetamine-containing urine (drug-free urine that was "spiked" with amphetamine ranging from 0.10 to 30.0 μg per 10 ml urine) specimens were prepared. The duplicates were then spotted on separate TLC plates, coated with a 250-μm layer of silica gel without fluorescent indicator. The TLC plates were developed in ethyl acetate:methanol:concentrated ammonium hydroxide (170:20:10 by volume) [*12*]. The plates were removed when the solvent migrated between 18 and 19 cm and dried with warm air from a hair drier type of air blower. Then the plates were dried 10 min in an oven at 75°C to remove residual ammonia. Residual ammonia interferes with the ninhydrin spray; it does not interfere with the fluorescamine reagent.

One TLC plate was developed with ninhydrin and the duplicate was developed with fluorescamine. When the fluorescamine test was applied to detection of amphetamine on thin-layer chromatograms, the fluorescamine-acetone solution (50 mg%) was first sprayed onto the dried chromatogram and then oversprayed immediately with the pH 9.0 borate buffer. The fluorescent produce was visualized and located with long-wave UV light (366 nm).

Results and Discussion

Theoretically, the fluorescamine test should yield positive results only with compounds containing a primary amine group. In order to verify this and evaluate possible interfering compounds, many common drugs which are seen as exhibits in the forensic chemistry laboratory were tested. The standard compounds contained in this laboratory consist of a combination of pure drugs, tablets, and capsules. The chemical constituents of the trade name compounds can be found in standard references [*13,14*]. About 10% of the standard compounds tested yielded a positive fluorescamine test; these results are contained in Table 1. From the molecular structures of the compounds examined, it was concluded that a primary amine was a requirement for obtaining a positive fluorescamine test.

Many compounds have native fluorescence; it is important to note this by determining the presence of fluorescence at the stage of addition of borate buffer, before the addition of fluorescamine. It is important to run blanks with this test because it is extremely sensitive and very small contamination will yield false positive results.

Amphetamine and methamphetamine can be distinguished easily by the fluorescamine test. Both of these compounds give the same colors in the Marquis, Meckes, and Froehdes spot tests, and the UV spectra of these compounds are indistinguishable. However, the fluorescamine test is positive with the primary amine, amphetamine, and negative with the secondary amine, methamphetamine.

Sometimes amphetamine preparations have caffeine or other components which interfere with the UV spectrum. Under this circumstance it is convenient to spot 2 to 3 μl of the positive fluorescamine spot test on a thin-layer chromatogram and compare with standard fluorescent derivatives. The chromatographic mobility of the fluorescent derivative of amphetamine has been found to be unique. No other standard compound (see Table 1) has been found to form a fluorescent derivative with fluorescamine and then migrate the same to that to amphetamine fluorescent derivative. The amino acids tyrosine, phenylalanine, and histidine have been studied, as well as Aldomet® (α-methyldihydroxyphenylalanine). All four of the fluorescent derivatives of these compounds remained at the origin in the chloroform:methanol (90:10) mobile phase, while the amphetamine fluorophore moved at an R_f of 0.30.

Often it is necessary to perform urinalysis to determine amphetamine abuse. The classic method of detection of amphetamine in urine is extraction and TLC analysis with ninhydrin spray. The generally accepted minimal detection limit of ninhydrin reagent spray for this purpose is 1 μg/ml of urine [*15*]. No detailed study of amphetamine extraction efficiency was performed because the mission of this study was determination of the relative detection

TABLE 1—*Examination of standard drugs with the fluorescamine test.*

Drug	Test	Drug	Test
1. Acetylcodeine	–	49. Caffeine (PCP)	–
2. Amesec® Caps (aminophylline compound)	–	50. Cafilon® (calcium lactate)	–
3. Aminophylline + Phenobarbital Tablets	–	51. Calcium cyclobarbital	–
4. Aminopyrine	–	52. Camoquin® HCl (amodiaquin)	–
5. Aminosalicylic acid	–	53. Camphocodine®	–
6. Amobarbital	–	54. Carbarsone® (N-carbamoylarsanilic acid)	+
7. *dl*-Amphetamine	+	55. Carbinoxamine maleate	–
8. *d*-Amphetamine sulfate	+	56. Carisoprodol	–
9. Sodium Amytal®	–	57. Cascara sagrada	–
10. Amphojel® (aluminum hydroxide)	–	58. Chloracetophenone	–
11. Ampicillin trihydrate	+	59. Chloramphenicol	–
12. Ampicillin	+	60. Chloromycetin® powder (chloramphenicol)	–
13. Anacin®	–	61. Chloral hydrate	–
14. Anahist®	–	62. Chloropyrilene	–
15. Analexin-AF®	–	63. Chloroquimediphosphate	–
16. Ananase® (plant protease concentrate)	–	64. Chloroquine phosphate	–
17. Ansional®	–	65. Chlorpheniramine	–
18. Antipyrine	–	66. Chlortetracycline HCl	–
19. Anturane®	–	67. Chlor-Trimeton® (chlorpheniramine maleate)	–
20. Apresoline® HCl (hydralazine HCl)	–	68. Cholan® (dehydrocholic acid)	–
21. Ascorbic acid	–	69. Chrysarobin	–
22. Atarax® (hydroxyzine HCL)	–	70. Cibalgin®	–
23. Atraxin® (meprobamate)	–	71. CKD Tablet® (ephedrine HCl)	–
24. Atropine sulfate	–	72. Clarmil	–
25. Auramin®	–	73. Cocaine	–
26. Aureomycin®	–	74. Codeine	–
27. Aventyl® HCl (nortriptyline HCl)	–	75. Colchicine	–
28. Barbital	–	76. Combid®	–
29. Belladona	–	77. Compazine®	–
30. Bellergal® Tablets	–	78. Co-Pyronil®	–
31. Bellergal® Spacetabs®	–	79. Cortancyl® (prednisone)	–
32. Benadryl®	–	80. Coricidin D®	–
33. Bendectin®	–	81. Cortisone acetate	–
34. Benemid® (probenecid)	–	82. Creatine	–
35. Benzedrine® (amphetamine sulfate)	+	83. Crystoids® (hexylresorcinol)	–
36. Benzocaine (ethyl aminobenzoate)	–	84. Crystoserpine® (reserpine)	–
37. Beta-Chlor® (chloral betaine)	–	85. Cyclandel	–
38. Bicillin® (benzathine penicillin G)	–	86. Cyclobarbital	–
39. Binoctal® (amobarbital, secobarbital)	–	87. Dalmane® (flurazepam HCl)	–
40. Bismuth subnitrate	–	88. Daprisal®	+
41. Bromanautine®	–	89. Dapsone® (4′, 4′-sulfonyldianaline)	+
42. Brovarin®	–	90. Darvon® (propoxyphene HCl)	–
43. Brucine	–	91. Darvon® Compound-65	–
44. Butabarbital	–	92. Darvo-Tran®	–
45. Butalbital	–	93. DBI® (phenformin HCl 5)	–
46. Butazolidin® (phenylbutazone)	–	94. Declomycin® (demethylchlortetracycline HCl)	–
47. Cafergot® (ergotamine tartrate and caffeine)	–	95. Delta-Cortef® (prednisolone)	–
48. Caffeine	–	96. Dexamyl®	+
		97. Dexedrine® (*d*-amphetamine sulfate)	+
		98. Diabinese® (chlorpropamide)	–
		99. Dianabol® (methandrostenolone)	–
		100. Diethylstilbestrol	–

TABLE 1—*Continued.*

Drug	Test	Drug	Test
101. Digitalis	–	150. High Grelan® (secopyrabital)	–
102. Digitoxin	–	151. Homatropine hydrobromide	–
103. Digoxin	–	152. Hygroton® (chlorthalidone)	–
104. Dihydrostreptomycin polymix	–	153. Hyminol® (methaqualone)	–
105. Diiodohydroxyquin	–	154. Hyoscyamine or stramonium	–
106. Dilantin® (diphenylhydantoin)	–	155. Ilosone® (erythromycin esto-	+
107. Dimetane® (brompheniramine maleate)	–	late)	
		156. Immenoctal® (amobarbital)	–
108. Dimetapp®	+	157. Indocin® (indomethacin)	–
109. Dioctyl sodium	–	158. Ismelin® sulfate (guanethidine	–
110. Diphenhydramine	–	sulfate)	
111. Diphenylhydantoin with pheno-	–	159. Isopropyl meprobamate	–
barbital		160. Isordil® (isosorbide dinitrate)	–
112. Disophrol Chronotab®	–	161. Kafe Soft® (caffeine)	–
113. Diuril®	–	162. Kemicetine® (chloramphenicol)	+
114. Doloran® (allobarbital)	–	163. Ketamine	–
115. Donnatal®	–	164. Leukeran® (chlorambucil)	–
116. Doriden® (glutethimide NF)	–	165. Librium® (chlordiazepoxide	–
117. Dormopan® (hexobarbital)	–	HCl)	
118. Doxidan®	–	166. Lidocaine HCl (injection, USP	–
119. Dramamine® (dimenhydrinate)	–	2%)	
120. Drixoral®	–	167. Lincocin® (lincomycin HCl	–
121. Ducolax® (bisacodyl)	–	monohydrate)	
122. Edrisal®	+	168. Lomotil® (diphenoxylate HCl	–
123. Elavil® HCl (amitriptyline	–	with atropine sulfate)	
HCl)		169. Lysergic acid diethylamide	–
124. Ephedrine HCl	–	170. Mandelamine® (methenamine	–
125. Ephedrine sulfate	–	mandelate)	
126. Ergotrate® maleate (ergonovine	–	171. Mannitol (Korean)	–
maleate)		172. Maple Tablet (maple prepara-	–
127. Erythrocin® (erythromycin	–	tion)	
ethylsuccinate)		173. MDA (methylenedioxyamphet-	+
128. Erythromycin-base Film Tab®	–	amine)	
129. Erythromycin stearate	–	174. Marezine® (cyclizine)	–
(Ilotycin®)		175. Meclizine	–
130. Erythromycin-tan®	–	176. Meconic acid	–
131. Eskatrol® Spansule®	+	177. Medroxyprogesterone acetate	–
132. Estinyl® (ethinyl estradiol)	–	178. Mellaril® (thioridazine)	–
133. Ethylmorphine	–	179. Mephenesin	–
134. Ferrous sulfate	–	180. Mephobarbital	–
135. Fiorinal®	–	181. Mepivacaine HCl injection 1%	–
136. Flagyl®	–	(carbocaine)	
137. Floraquin® vaginal tablets	+	182. Meprobamate	–
(diiodohydroxyquin)		183. Meprophen® (meprobamate)	–
138. Flurazepam HCl	–	184. Meridon	–
139. Gantrisin® (sulfisoxazole)	+	185. Merthiolate® (thimerosal)	–
140. Glutethimide	–	186. Methadone	–
141. Grifulvin V® (griseofulvin mi-	–	187. Methamphetamine	–
crosize)		188. Methapyrilene	–
142. Griseofulvin	–	189. Methaqualone	–
143. Gynergen® (ergotamine	+	190. *dl*-Methionine and vitamin B_1	+
tartrate)		191. Methylcellulose	–
144. Halotestin® (fluoxymesterone)	–	192. Methylhexabarbital	–
145. Hashish	–	193. 3-Monoacetylmorphine	–
146. Helozid	–	194. Mycostatin® (nystatin USP)	+
147. Heptabarbital	–	195. Myleran® (busulfan)	–
148. Heroin (diacetylmorphine)	–	196. Mylicon®	–
149. Hetrazan® (diethylcarbamazine	–	197. Mysoline® (primidone)	–
citrate)		198. Naron® (cyclopyrabital)	–

TABLE 1—*Continued.*

Drug	Test	Drug	Test
199. NegGram® (nalidixic acid)	–	247. Promacetin® (acetosalfone sodium)	–
200. Nembutal® (sodium pentobarbital)	–	248. Pronestyl® (procainamide HCl)	–
201. Neomycin	+	249. Propadrine® (phenylpropanolamine)	+
202. Nicotinic acid	–	250. Propadrine HCl®	+
203. Nitrofurantoin	–	251. Propoxyphene HCl	–
204. Nitroglycerin	–	252. Prostaphlin® (sodium oxacillin)	–
205. Norflex® (orphenadrine citrate)	–	253. Provest® Daypak (birth control)	–
206. Norgesic®	–	254. Pyridium® (phenazopyridine HCl)	–
207. Norinyl® (norethindrone with mestranol)	–	255. Pyridoxine HCl (vitamin B_6)	–
208. Noludar® (methyprylon)	–	256. Quinacrine HCl	–
209. Novahistine® LP tablets	–	257. Quinidine sulfate	–
210. Novatophen® (neocinchophen)	–	258. Quinine sulfate	–
211. Optalidon® (allyisobutylbarbital)	–	259. Rarical	–
212. Opium (raw)	–	260. Rela® (carisoprodol)	–
213. Oretic® (hydrochlorthiazide)	–	261. Rhubarb	–
214. Ornade® Spansule®	+	262. Riboflavin (vitamin B_2)	–
215. Oxsoralen® (methoxsalen)	–	263. Ritalin® HCl (methylphenidate HCl)	–
216. Paraflex® (chlorzoxazone)	–	264. Robaxin® (methocarbamol)	–
217. Pavatrine® HCl	–	265. Romilar® (racemethoxphan HBr)	–
218. Pavron®	–	266. Rotoxamine	–
219. Penicillin G potassium	–	267. Salol® (phenyl salicylate)	–
220. Penicillin, phenoxymethyl potassium	–	268. Sand	–
221. Pentobarbital	–	269. Sansert® (methysergide maleate)	–
222. Periactin® HCl (cyproheptadine HCl)	–	270. Saridon® (isopropyl antipyrine)	–
223. Peritrate® (pentaerythritol tetranitrate)	+	271. Scopalamine hydrobromide	–
224. Persantine® (dipyridamole)	–	272. Secobarbital	–
225. Phenaphen®	–	273. Sedes® (hexobarbital)	–
226. Phencyclidine	–	274. Sedalin® (pyrabital)	–
227. Phenergan®	–	275. Sernylan® (phencyclidine HCl)	–
228. Phenformin HCl 5	–	276. Sinequan (10 mg)	–
229. Phenobarbital	–	277. Sintrom® (acenocoumarol)	–
230. Phenobarbital, ephedrine, and theophylline	–	278. Sodium bicarbonate	–
231. Phenylpropanolamine HCl	+	279. Sodium chloride and sodium bicarbonate	–
232. Physostigmine salicylate	–	280. Sodium salicylate	–
233. Pilocarpine nitrate	–	281. Sparine® (pronazine HCl)	–
234. Pival® (pindone)	–	282. Stelazine® (trifluoperazine)	–
235. Placidyl® soft capsules (ethchlorvynol)	–	283. Streptomycin	+
236. Polycillin® (ampicillin trihydrate)	–	284. Strychnine	–
237. KMnO® tablet	–	285. Sudafed® (pseudoephedrine HCl)	–
238. Povan® (pryvinium pamoate)	–	286. Sulfadiazine	–
239. Prednisolone	–	287. Surfak® [calcium bis-(dioctyl sulfosuccinate)]	–
240. Prednisone	–	288. Syntho Tab	–
241. Preludin® (phenmetrazine HCl)	–	289. Surgex® (pipradral)	–
242. Premarin®	–	290. Talwin® (pentazocine)	–
243. Pre-Sate® (chlorphentermine HCl)	–	291. Tapazole® (methimazole)	–
244. Primaquine phosphate	–	292. Tenuate® Dospan® (diethylpropion HCl)	–
245. Pro-Banthine® (propantheline bromide)	–	293. Terramycin® (oxytetracycline)	–
246. Procaine hydrochloride	+	294. Tessalon® (benzonatate)	–

TABLE 1—*Continued.*

Drug	Test	Drug	Test
295. Tetracycline	–	311. Tylenol® (acetaminophen)	–
296. Tetrex® (tetracycline phosphate)	–	312. Urecholine® (bethanechol chloride)	–
297. Thebaine	–	313. Valium® (diazepam)	–
298. Theophylline	–	314. Vallestril® (methallenestril)	–
299. Thianphenicol	–	315. Vasodilan® (isoxsuprine HCl)	–
300. Thorazine® (chlorpromazine)	–	316. Vigosan®	–
301. Tigan® (trimethobenzamide HCl)	+	317. Vistaril® (hydroxyzine pamoate)	–
302. Titralac® (calcium carbonate)	–	318. Vitamin B_1	+
303. Tofranil® (imipramine HCl)	–	319. Vitamin K (synthetic)	–
304. Tranquinal® (meprobamate)	–	320. Warfarin	–
305. Triacetyloleandomycin	+	321. Wyamine sulfate® (mephentermine)	–
306. Trilafon® (perphenazine)	–		
307. Tri-Span®	+	322. Wyanoids®	–
308. Tropacocaine HCl	–	323. Zactirin®	–
309. Tuinal® (sodium amobarbital and sodium secobarbital)	–	324. Zarontin® (ethosuximide)	–
		325. Zyloprim (allopurinol)	–
310. Tuss-Ornade®	–		

limit of ninhydrin and fluorescamine. It has been found that fluorescamine is a more sensitive method for the detection of amphetamine on thin-layer chromatograms. These results are tabulated in Table 2. The detection limit of amphetamine in urine samples is increased 100 times when the only parameter varied is the method of detection, ninhydrin or fluorescamine.

Other physiological chemicals containing primary amino groups normally present in drug-free urine extracts will also react with fluorescamine. This was evidenced in the non-drug-containing urine (Table 2). As many as eight well-separated fluorescent areas were routinely observed. These naturally occurring substances containing primary amino groups all have mobilities considerably less than that of amphetamine in the developing solvent system used; thus, the naturally occurring compounds do not interfere with the analysis.

While this paper was in the process of publication another has appeared on the use of fluorescamine in amphetamine detection in urine [*16*]. These authors have also stressed the increased detection limit of fluorescamine compared to ninhydrin. Klein et al quantitated the amphetamine level by extracting the silica gel area containing the amphetamine fluorophore and analyzed the extract in a microfluorimeter. They also reported that after amphetamine was made visible by fluorescamine spray, the plate could be sprayed with other common identification reagents with no interference. Accordingly, it is possible to substitute fluorescamine for ninhydrin in a routine battery of sprays for drug abuse screening in urine samples.

Summary

This paper describes some applications of the fluorescamine spot test to forensic toxicological analysis. The fluorescamine test only reacts with primary amines; thus, this test makes a clear-cut distinction between amphetamine and methamphetamine. Previous common spot tests used reacted the same with these two amines. Fluorescamine is 100 times more sensitive in detecting amphetamine extracted from urine on thin-layer chromatograms than ninhydrin. Thus, it is a more sensitive method of detecting amphetamine abuse in urinalysis screening programs.

TABLE 2—*Relative detection of amphetamine isolated from urine with ninhydrin and fluorescamine on thin-layer chromatograms.*

Amphetamine[a], μg	Ninhydrin	Fluorescamine
30.0	+	+
20.0	±	+
10.0	−	+
5.0	−	+
0.5	−	+
0.25	−	+
0.1	−	±
0	−	−

[a]Amount of amphetamine added to 10 ml of control, non-drug-containing urine.

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