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Short Communication

Quantification of amphetamine in urine: solid-phase extraction, polymeric-reagent derivatization and reversed-phase high-performance liquid chromatography

Daniel H. Fisher*

Department of Medical Laboratory Science, Northeastern University, Boston, MA 02115 (USA)

Andre J. Bourque

Department of Chemistry and Barnett Institute, Northeastern University, Boston, MA 02115 (USA)

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ABSTRACT

The concentration of amphetamine was determined in urine using solid-phase extraction, polymeric-reagent derivatization, and reversed-phase high-performance liquid chromatography wilt ultraviolet detection. To remove a majority of acidic and neutral compounds in urine, a solid-phase extraction was first performed on a sample spiked with the internal standard, 1-methyl-3-phenylpropylamine. Because amphetamine has a relatively low molar absorptivity, the base was derivatized with a polymeric 1-hydroxybenzotriazole reagent containing a 3,5-dinitrobenzoate active ester. The limit of detection is 14 ng/ml, and the limit of quantification is 47 ng/ml. The calibration curve is linear from 0.01 to 4.0 μg/ml. The pooled relative standard deviation is ± 5.5% for eight urine samples measured in duplicate. The average relative error (bias) is +2.2% when compared to gas chromatography–mass spectrometry.

INTRODUCTION

Our goal was to develop a high-performance liquid chromatographic (HPLC) method for determining urine amphetamine concentrations as part of the process of adapting immunoassay reagents to an automated chemistry analyzer given the following constraints. (1) The urine volume must not exceed 2 ml to allow enough sample for duplicate analysis of expensive immunoassay urine standards. (2) The limit of detection must be below 25 ng/ml in order to quantitate the lowest expected concentrations in the urine samples. (3) The procedure had to be amenable to batch analysis, because of the large number of urine samples to be analyzed. (4) To contain costs, the equipment was limited to a gradient solvent system, a variable-wavelength UV detector and a conventional (4 mm I.D.) reversed-phase column.

HPLC methods based on ultraviolet absorption of amphetamine using a conventional column [1,2] and a microbore (1 mm) column have been reported [3]. Amphetamine can also be de-

* Corresponding author.

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rivatized with a chromophore or fluorophore using a reagent dissolved in solution [4,5] or a reagent covalently bonded to a polymeric support [6–11]. However, none of these previously reported HPLC methods meet all of our constraints.

We report here a batch method for quantification of amphetamine in spiked human urine samples that meets these constraints. The method described in this paper is based on previously reported work using direct injection of urine, online polymeric-reagent derivatization, a conventional column and ultraviolet detection [6]. A mixed-mode solid-phase extraction was employed to reduce endogenous interference in urine before derivatization with the polymeric benzotriazole-3,5-dinitrobenzoate reagent. Instrumentation Laboratory used the data from this work as part of the process of adapting the Syva EMIT reagents to their automated chemistry analyzer, the Monarch [12]. This method could be used for similar studies.

EXPERIMENTAL

Materials

Water (HPLC grade), methanol (HPLC grade), 30% ammonium hydroxide (reagent grade), potassium hydroxide (reagent grade), monobasic potassium phosphate (HPLC grade), acetonitrile (HPLC grade, absorbance at 220 nm = 0.004) and ethyl acetate (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade glacial acetic acid was supplied by J.T. Baker (Phillipsburg, NJ, USA). Triethylamine (99+%, Aldrich, Milwaukee, WI, USA) must be of the highest purity available in order to decrease the reagent blank at 220 nm. *d,l*-Amphetamine sulfate was obtained from Sigma (St. Louis, MO, USA), and *d,l*-1-methyl-3-phenylpropylamine [$C_6H_5CH_2CH_2CH(CH_3)NH_2$] from Aldrich. Dinitrobenzoylamphetamine and dinitrobenzoylbenzotriazole polymeric reagent were synthesized in house as previously reported [6]. Solid-phase extraction was done using a solid-phase extraction manifold (Supelco, Bellefonte, PA, USA) and Bond Elut Certify columns (130 mg, Varian Instruments, Harbor City, CA, USA). DAU I–IV urine controls were obtained from Ciba-Corning (Irvine, CA, USA) and the Sentry urine control was supplied Hycor Biomedical (Garden Grove, CA, USA). Level 1–5 urine controls were prepared by spiking pooled drug-free human urine (Instrumentation Laboratory, Lexington, MA, USA). This pooled urine was negative for amphetamines by EMIT.

High-performance liquid chromatography

A 600E multi-solvent delivery system (Waters, Milford, MA, USA) was set to 10 m*M* pH 2.5 phosphate buffer–acetonitrile (55:45, v/v) and 0.7 ml/min. A Waters U6K injector with a 200-μl loop was used. Separations were performed using a 10 mm × 4.6 mm I.D. Microsorb column (Rainin Instrument, Woburn, MA, USA) packed with octadecyldimethylsilyl 5-μm silica gel and a 2-cm Uptight precolumn (UpChurch Scientific, Oak Harbor, CA, USA) hand-packed with the octadecyldimethylsilyl 5-μm silica gel. A Spectra-100 variable-wavelength detector (Spectra-Physics, San Jose, CA, USA) was set to 220 nm. Chromatograms were recorded with a Spectra-Physics SP4270 integrator.

Standards

A 1.00 mg/ml solution of amphetamine in HPLC-grade water was prepared from the sulfate. The stock solution was used to prepare 0.50, 1.00, 2.00, and 4.00 μg/ml standards of amphetamine in freshly voided drug-free human urine. A 10 μg/ml solution of 1-methyl-3-phenylpropylamine, the internal standard, in methanol was prepared from a 1.00 mg/ml stock solution. All standards were stored at 4°C except during use. All standards were kept at room temperature long enough to dissolve any precipitate.

Extraction

Add 800 μl of a 100 m*M* pH 6.0 phosphate buffer and 200 μl of a 10 μg/ml solution of 1-methyl-3-phenylpropylamine to 2.0 ml of urine. Check that the pH is between 5 and 7 using pH paper. Adjust the pH as required using 1.0 *M* potassium hydroxide or 1.0 *M* HCl. Condition the Bond Elut Certify column with 3 ml of meth-

anol and 3 ml of the 100 m*M* pH 6.0 phosphate buffer. Do not allow the column to go dry. Extract the entire volume at a flow of four to six drops per second using about 0.5–0.7 kPa of vacuum. Stop the flow when the meniscus reaches the top of the bed. Wash with 1 ml of 1.0 *M* acetic acid followed by 3 ml of HPLC-grade water. Dry for 5 min under a vacuum of 2.0 kPa. Wash with 6 ml of methanol. Dry for 2 min under a vacuum of 2.0 kPa. Elute into a clean 125 mm × 16 mm disposable borosilicate glass test tube with 2 ml of a 2% (v/v) solution of 30% NH_4OH in ethyl acetate at a rate of four to six drops per second under 0.5–0.7 kPa of vacuum. Evaporate for 2 min under a gentle stream of nitrogen. Add 100 μl of 1.0 *M* HCl in diethyl ether. Evaporate under a stream of nitrogen at 40°C until dry.

Derivatization

Reconstitute the extraction residue in 125 μl of a solution containing triethylamine, water, and acetonitrile (1.4:8.6:90, v/v). Add the entire volume to 50 ± 5 mg of the dinitrobenzoylbenzotriazole polymeric reagent in a 1-ml disposable plastic pipet tip plugged with Kimwipe (a low lint, single ply, non-abrasive tissue). Weigh fresh polymeric reagent for each derivatization. React for 30 s and elute with 500 μl of acetonitrile under positive pressure. Inject 10–20 μl into the HPLC column.

RESULTS AND DISCUSSION

Extraction

The protocol reported here is similar to the protocol developed for gas chromatography–mass spectrometry (GC–MS) by Varian Instruments, the manufacturer of the Bond Elut Certify columns. The amount of urine sample was decreased from 5 to 2 ml and the amount of buffer was decreased proportionally in order to accommodate smaller sample sizes. The column was conditioned with 1.5 times the amount of methanol and phosphate buffer in order to ensure adequate conditioning. The flow-rate was carefully controlled during the extraction and elution steps. This provided a consistently high recovery (99%). After the acetic acid wash, the column was washed with 3 ml of HPLC-grade water. Without this step there was a significant precipitate upon the addition of ethereal HCl. Because amphetamine is a volatile liquid the HCl salt was formed by the addition of ethereal HCl. This solution was added after several minutes of evaporation in order to minimize the formation of an ammonium chloride precipitate. Up to twelve solid-phase extractions and derivatizations can be performed simultaneously in 1 h using a Supelco manifold. The samples could then easily be injected using an autoinjector. Therefore, 84 samples could be processed within a day.

Derivatization

The percentage derivatization is a function of the polymeric reagent, acetonitrile concentration, triethylamine concentration and time as has been previously reported [6]. Acetonitrile wets the polymer and provides the greatest percentage derivatization for a large number of amines. The derivatization of *n*-butylamine using a less activated polymeric reagent (*o*-nitrobenzophenol) is 91.5% in the presence of equimolar amounts of triethylamine, but only 56.3% in the absence of triethylamine. The derivatization of *n*-butylamine by the benzotriazole reagent reaches a maximum of 91.5% by 7 s compared to 40 s for the *o*-nitrobenzophenol reagent. Therefore, the polymeric benzotriazole-3,5-dinitrobenzoate reagent was selected (Fig. 1). The average relative error for a single-blind chiral analysis of amphetamine from 25–75 μg/ml in human urine is 2.6% ($n = 10$), and the pooled relative standard deviation is 0.8% (for ten samples derivatized in quadruplicate) as previously reported [6]. This reagent works equally well for both stereoisomers [6]. The amphetamine derivative can be detected at 254 nm although 220 nm is also satisfactory given the extensive sample clean-up.

After solid-phase extraction, the percentage derivatization of amphetamine is essentially zero without the addition of thiethylamine. This is probably due to residual acetic acid, HCl or both. Water is essential in order to dissolve the residue.

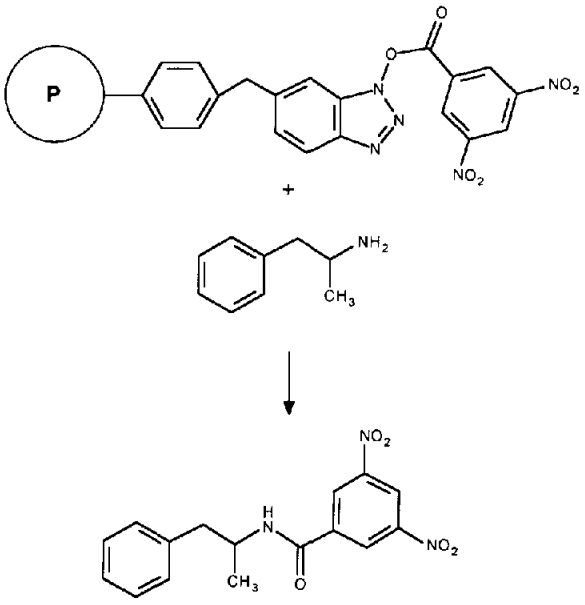

Fig. 1. Derivatization of amphetamine using the dinitrobenzoylbenzotriazole polymeric reagent.

The percentage derivatization of a simulated solid-phase extraction residue is 72%.

Molar absorptivity

The molar absorptivity of amphetamine is 492 cm^{-1} M^{-1} at 220 nm and 202 cm^{-1} M^{-1} at 254 nm. However, the molar absorptivity of 3,5-dinitrobenzoylamphetamine is 20 730 cm^{-1} M^{-1} at 220 nm and 10 800 cm^{-1} M^{-1} at 254 nm. The molar absorptivity of the derivative at 220 nm is forty times greater than that of amphetamine and fifty times greater at 254 nm. The amphetamine derivative can be detected at 254 nm, although 220 nm is also satisfactory given the extensive sample clean-up coupled with derivatization.

Linearity

The peak-area ratio was calculated by dividing the amphetamine area by the internal standard area. The standard curve was constructed by plotting peak-area ratio *versus* concentration of amphetamine. The calibration curve is linear from 10 ng/ml to 4.0 μg/ml (r = 0.999, slope = 1.17, y-intercept = −0.02).

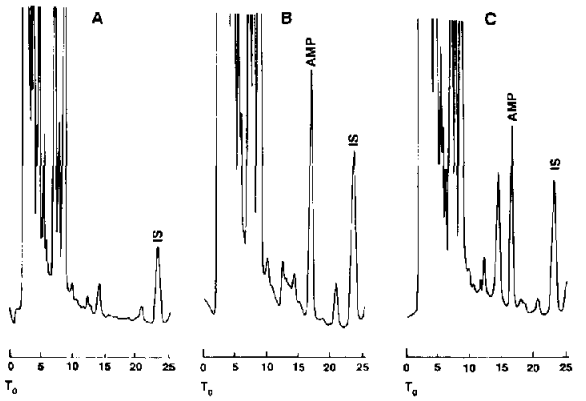

Fig. 2. Chromatograms of (A) urine blank and internal standard, (B) amphetamine urine standard (1.0 μg/ml) and internal standard, and (C) Ciba-Corning urine control DAU III (0.89 μg/ml) and internal standard. The retention time of the dinitrobenzoyl derivative of amphetamine and the internal standard is 16.3 and 23.0 min, respectively. Peaks: AMP = amphetamine; IS = internal standard.

Interference

No endogenous interfering peaks were observed in a blank urine specimen (Fig. 2). None of the eighteen additional basic drugs or potential derivatives in the Ciba-Corning DAU I–IV controls coeluted with 3,5-dinitrobenzoylamphetamine or internal standard: benzoylecgonine, cocaine, codeine, ecgonine methyl ester, gluthethimide, imipramine, meperidine, methadone and its metabolite, methamphetamine, methaqualone, morphine, morphine-3-glucuronide, nortriptyline, oxazepam, phencyclidine, propoxyphene, and quinine. This selectivity is based on limited access to the analytical reagent, as well as the removal of some of these species during the solid-phase extraction.

Detection and quantification limits

The limit of detection for amphetamine in human urine is 14 ng/ml and the limit of quantification is 47 ng/ml [13] for the method reported here. This method has a limit of detection well below the limit of detection of 0.3 μg/ml reported by Binder *et al.* [1] and the limit of detection of 25 μg/ml reported by Bourque and Krull [6]. Both Binder *et al.* and Bourque and Krull used a conventional HPLC column and ultraviolet detec-

TABLE I

AMPHETAMINE CONCENTRATION IN THE URINE CONTROLS

Sample	Concentration (μg/ml)		
	Found	S.D.	Expected
Instrumentation Laboratory level 1	< LOQ[a]	N.A.[b]	Negative
Instrumentation Laboratory level 2	0.44	0.00	0.50[c]
Instrumentation Laboratory level 3	0.67	0.01	0.75[c]
Instrumentation Laboratory level 4	1.02	0.01	1.00[c]
Instrumentation Laboratory level 5	1.34	0.08	1.27[c]
Ciba-Corning DAU I	< LOQ[a]	N.A.[b]	Negative
Ciba-Corning DAU II	0.42	0.03	0.40[d]
Ciba-Corning DAU III	0.89	0.03	0.85[d]
Ciba-Corning DAU IV	1.89	0.10	2.00[e]
Hycor Sentry	1.21	0.13	1.25[d]

[a] Limit of quantification.

[b] Determination of standard deviation in this case is not applicable because the concentration is below the limit of quantification.

[c] Concentration calculated from the amount of amphetamine added to drug-free human urine.

[d] Concentration determined by GC–MS for the vendor.

[e] Target concentration for the control.

tion. The limit of detection is similar to the 20 ng/ml value reported by Slais *et al.* [3] using a microbore column and ultraviolet detection. Maeder *et al.* [11] reported a limit of detection of 2.7 ng/ml using on-line derivatization, a conventional HPLC column and ultraviolet detection; however, this was not for urine samples but for aqueous standards. The work of Bourque *et al.* [6] indicates that on-line derivatization of amphetamine in urine samples results in a high sample blank when ultraviolet detection is used. When amphetamine is derivatized via an on-line polymeric mixed-bed reactor, the peak height of the 9-fluorenylmethoxycarbonyl and the dinitrobenzoyl derivative are similar [8].

Accuracy and precision

The results of the external quality control samples analyzed using the protocol reported here are summarized in Table I. Each sample was analyzed in duplicate. The expected value for Instrumentation Laboratory level 1–5 and Ciba-Corning DAU I and IV urine controls is based upon the calculated concentration. The expected value for the Ciba-Corning DAU II, DAU III, and Hycor Sentry urine controls are actual values obtained by GC–MS. The relative error (bias) for DAU II, DAU III, and Sentry urine controls is +5.0%, +4.7% and −3.2%, respectively. The average relative error is +2.2%. The pooled relative standard deviation is ±5.5%. (n = 16) for level 2–5, DAU II–IV, and Sentry urine controls.

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