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Determination of d-amphetamine in biological samples using high-performance liquid chromatography after precolumn derivatization with *o*-phthaldialdehyde and 3-mercaptopropionic acid

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Abstract

An HPLC method is described for the determination of amphetamine using fluorometric detection after derivatization with *o*-phthaldialdehyde and 3-mercaptopropionic acid. This procedure is more sensitive (detection limit 370 fmol in microdialysate buffer standards, 1.5 pmol in extracted plasma and tissue samples) than most of the previous methods described for the determination of amphetamine with HPLC-fluorescence detection. Due to the stability of the derivative it is also suitable for autosampling after manual derivatization. Investigators currently using *o*-phthaldialdehyde derivatization and fluorometric detection for amino acid determination should be able to rapidly implement this method.

1. Introduction

Chromatographic methods for the quantitation of amphetamine (AMPH) in tissues and body fluids are known for more than two decades and include gas chromatography [1], gas–liquid chromatography [2] and combined gas chromatography–mass spectrometry (GC–MS) [3]. Today, with GC–MS minimum AMPH concentrations as low as 0.1 ng/ml can be quantitated [4]. However, HPLC often is an alternative, when mass fragmentation is not an analytical requirement, which is less expensive and simpler yet sensitive enough for many applications in biomedical research. The determination of AMPH by HPLC has been utilized for 20 years [5]. AMPH was quantitated after derivatization by UV [6–8], fluorescent [9–11], chemiluminescence [12], and more recently by diode-array UV [13] detection. For laboratories without access to a chemiluminescent or photodiode-array detector, fluorescent detection frequently is the method of choice for the quantitation of primary amines. In this context, *o*-phthaldialdehyde (OPA) is the most widely reported derivatizing reagent for HPLC quantitation of primary amines, with either electrochemical, UV, or fluorescence detection [14].

Over decades, AMPH and related compounds have attracted attention in pharmacology and neuroscience because of their neurotoxic potential and use as a neuropharmacological tool in

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the study of axon terminal degeneration or functional regulation of the dopaminergic neurons of the nigro-striatal pathway [15]. The neurotoxic effects of amphetamines like AMPH or methamphetamine can be greatly potentiated by hyperthermia in both rat [16] and mouse [17]. Questions have arisen as to whether increased brain levels of amphetamines which occur either during hyperthermia or due to the combined administration with other drugs are responsible for the appearance of neurotoxicity. The purpose of this study was to develop a method for the determination of AMPH in microdialysate, plasma and tissue extracts using sensitive HPLC fluorescence detection of derivatives stable enough for an autosampler procedure. This was achieved by utilizing 3-mercaptopropionic acid (3-MA) instead of β-mercaptoethanol which was frequently used by others for derivatization. In contrast to β-mercaptoethanol, 3-MA is less frequently used in amino acid analysis [18,19], and has not yet been applied to the determination of amphetamine [20].

2. Experimental

2.1. Chemicals

L-Arginine hydrochloride (99 + %), d-amphetamine sulfate, benzylamine hydrochloride, glutamic acid (99 + %), L-lysine monohydrochloride (99 + %), 2-mercaptoethanol, *o*-phthaldialdehyde (99%), phosphoric acid and tryptamine (99 + %) were obtained from Sigma (St. Louis, MO, USA). Ethanol (200 proof) was obtained from Midwest Grain Products (Weston, MO, USA). (+)-4-Hydroxyamphetamine hydrobromide (99.2%) was obtained from NIDA Drug Supply (Rockville, MD, USA). 3-Mercaptopropionic acid (99 + %) was obtained from Aldrich (Milwaukee, WI, USA). Calcium chloride, dibasic potassium phosphate, D-glucose, hydrochloric acid, magnesium chloride, methanol, potassium chloride, potassium hydroxide, sodium chloride, sodium hydroxide, and ethyl acetate were obtained from J.T. Baker (Phillipsburg, NJ, USA). Sodium tetraborate decahydrate (99.5%) was obtained from Fluka (Buchs, Switzerland). Purified water from a Milli-Q water system (Millipore Corp., Marlborough, MA, USA) was used for the preparation of buffers, standards, and samples.

2.2. Equipment

A Waters HPLC system (Waters Associates, Milford, MA, USA) was used and included the following components: two Model 510 pumps controlled by a Model 680 gradient controller, a Model 470 scanning fluorescence detector, and a Model 746 data module integrator. Chromatographic separation was performed with a Supelco LC-18 250 × 4.6 mm I.D. analytical column, 5 μm particle size, (Supelco, Bellefonte, PA, USA). A C-130B guard column, 20 × 2 mm I.D. (Upchurch Scientific, Oak Harbor, WA, USA), packed with Bondapak C_{18}/CORASIL, 37–50 μm particle size (Waters Associates, Milford, MA, USA) was placed prior to the analytical column. This HPLC system was connected to a Biorad AS-100 HRLC automatic sampling system (Biorad Laboratories, Hercules, CA, USA). Brain tissue was disrupted by ultrasonication with a L&R transistor/ultrasonic T-14 (L&R manufact., Kearny, NJ, USA). For centrifugation a Hermle Z 360 K centrifuge (Gosheim, Germany) was used.

2.3. Collection of body fluids and brain samples

Microdialysis samples were collected as described elsewhere [21], however the composition of the microdialysis buffer was slightly changed to: NaCl 145 m*M*, KCl 1.5 m*M*, $MgCl_2 \cdot 6H_2O$ 1.5 m*M*, $CaCl_2 \cdot 2H_2O$ 1.25 m*M*, glucose 10 m*M*, K_2HPO_4 1.5 m*M*, (adjusted to pH 7.0 with HCl). For the collection of plasma, rats were decapitated and trunk blood was collected into heparin-coated glass tubes. Blood samples were centrifuged under refrigeration for 10 min at 18 000 *g*. Brains were quickly removed from the skull and dissected on a chilled glass plate. Both striata were collected. Samples of brain tissue and blood were also collected from untreated

animals for blanks and standards. All samples were stored at −150°C until analysis.

2.4. Preparation of OPA reagent

For fluorescence detection the OPA reagent was prepared as described by Joseph and Marsden [22], with the modification that 3-MA was used instead of 2-mercaptoethanol. After dissolving 27 mg of OPA in 500 μl of ethanol, 5 ml of 0.1 *M* borate buffer (pH 9.6) and 40 μl of 3-MA were added. This mixture was kept refrigerated and was used for up to four consecutive days. For the purpose of comparison in some analyses, 2-mercaptoethanol was used instead of 3-mercaptopropionic acid, which was added to the OPA–ethanol–borate solution (see above) either in a volume of 30 μl or 300 μl.

2.5. Sample preparation and extraction procedures

Microdialysis samples were injected directly after derivatization with 20 μl of OPA reagent.

Plasma samples were extracted under the following basic conditions. A 100-μl volume of 0.1 *M* borate buffer (adjusted to pH 10.6 with sodium hydroxide) and 200 μl of ethyl acetate were added to a 45-μl aliquot of plasma plus 5 μl of an aqueous solution containing an internal standard. The mixture was vortex-mixed for 2 min. After 10 min on ice an additional 200 μl of ethyl acetate and 200 μl of water were added to the mixture followed by brief vortex-mixing to further facilitate extraction. Centrifugation for 10 min at 18 000 *g* and 4°C was used to separate the organic and aqueous phases. Then 200 μl of the organic supernatant was transferred to a glass tube and dried at 45°C under nitrogen. Samples were reconstituted in 80 μl of KH_2PO_4 (0.05 *M*, adjusted to pH 2.6 with phosphoric acid). After adding 20 μl of borate buffer (adjusted to pH 11.5 with sodium hydroxide) samples were derivatized with 20 μl of the OPA reagent. To the remaining mixture of plasma, ethyl acetate and water an additional volume of 200 μl of ethyl acetate was added for a second extraction and subjected to the procedure described above.

Brain samples were weighed and diluted with 9 volumes of 0.1 *M* borate buffer (pH 10.6) containing defined concentrations of internal standard. After 20 s of ultrasonication, the samples were centrifuged for 10 min at 18 000 *g* and 4°C. AMPH was extracted from 100 μl of the supernatant using the same procedure as for plasma.

Tryptamine (TRP) was used as internal standard with either 5 μl of 40 μ*M* added to the sample in case of plasma (providing a theoretical concentration of 2.0 μ*M* in the injected sample) or, in case of brain samples, 8 μ*M* TRP was contained in the borate buffer that was added before sonication (providing a theoretical concentration of 3.6 μ*M* in the injected sample).

OPA reagent always was added to the sample at least 2 min before injection. Derivatization was performed in capped Eppendorf tubes which were kept at 4°C until injection.

2.6. Chromatographic conditions

A linear gradient program (Table 1) was run at ambient temperature with mobile phase A consisting of 65% KH_2PO_4 (0.05 *M*, adjusted to pH 5.5 with potassium hydroxide) and 35% methanol, and mobile phase B with inverse proportions of buffer (35%) and methanol (65%). The buffer solution was filtered after

Table 1
Chromatographic linear gradient conditions for HPLC determination of OPA–3-mercaptopropionic acid/amphetamine derivatives

Time (min)	Flow-rate (ml/min)	Buffer A (%)	Buffer B (%)
Initial (0)	1.5	100	0
0–3	1.5	100	0
4	1.5	0	100
18	1.5	0	100
19	1.5	100	0
25	1.5	100	0

Table 2
Retention times and sensitivities relative to amphetamine for other primary amines spiked into microdialysate buffer at 2 μM

Compound	Retention times (min before AMPH)	Sensitivity (mean ± S.E.M.) (% relative to AMPH)
Glutamic acid	12.67	214 ± 2.1
Arginine	11.78	107 ± 0.4
Lysine	3.27	100 ± 1.2
p-OH-Amphetamine	2.48	192 ± 0.3
Benzylamine	1.72	11 ± 0.3
Tryptamine	1.27	139 ± 2.6

preparation and the mobile phases were degassed for 90 s before use. Fluorescent detector settings were: $\lambda_{excitation}$ 340 nm, $\lambda_{emission}$ 440 nm, attenuation 8, gain 10×, filter 1.5 s. The autosampler was equipped with a 1000-μl loop, and its settings were: sample tray temperature 4°C, fill-speed: 1.0 ml/min, sample volume 75 μl, overfill-volume 50 μl.

2.7. Assay validation

The intra- and inter-assay precision (expressed as relative standard deviation) and accuracy (bias from nominal standard concentration) were assessed for AMPH spiked microdialysis buffer. Intra-assay validation was performed at different concentrations with four replicates for each concentration. The same concentrations were used for inter-assay validation. Duplicate analyses on four different days were performed for this purpose.

3. Results and discussion

3.1. Rationale for chosen method

The method described here for the assay of AMPH was developed because it could be rapidly adapted from similar methods for determining amino acids using OPA and fluorometric detection as originally described by Roth [23] and widely used since then, because of its sensitivity and simplicity [14]. 3-MA was chosen over 2-mercaptoethanol for OPA derivatization because the derivatized product proved to be more polar and more stable than the product from reacting with 2-mercaptoethanol–OPA. Derivatization of

Table 3
Stability of OPA–3-mercaptopropionic acid derivatives

Compound	Percent of immediately analyzed samples (mean ± S.E.M.)				
	1 h	2 h	6 h	12 h	24 h
AMPH	93 ± 3.4	99 ± 1.3	82 ± 3.2[a]	82 ± 5.4[a]	75 ± 5.6[a]
*p*OH-A	93 ± 3.7	100 ± 1.1	84 ± 3.3[a]	85 ± 5.7[a]	76 ± 13.2[a]
TRP	88 ± 3.5	87 ± 5.0	66 ± 4.3[a]	50 ± 3.3[a]	25 ± 2.9[a]

Samples were stored after derivatization of standards containing d-amphetamine, *p*-hydroxyamphetamine and tryptamine for the indicated time at 4°C in the autosampler ($n = 6$ per time point, final concentrations corresponding to 0.5 μM).
[a] Significantly lower than fresh prepared reference samples ($p \leq 0.05$).

AMPH with 2-mercaptoethanol–OPA results in a less polar product with retention time of 21.2 min under the chromatographic conditions used. In addition, the fluorescence intensity of the 2-mercaptoethanol derivatives was 8–10 fold lower as compared to 3-MA derivatization. This was not improved by increasing the amount of 2-mercaptoethanol in the OPA solution from 30 to 300 μl.

3.2. Selection of internal standard

TRP was chosen as an internal standard because (1) its retention time was longer than that of all amino acids and *p*-hydroxyamphetamine (p-OHA) but shorter than AMPH, (2) its percent recovery from brain and plasma was close to that of AMPH, (3) its fluorescence intensity with 3-MA was about the same as for AMPH and (4) it was not present in detectable quantities in blank brain and plasma samples. It is known that TRP in rat brain has a very short half life (0.3–0.7 min) and is synthesized only at a rate of 0.1–0.2 nmol/g/h [24]. Although the retention time of benzylamine was appropriate, its fluorescent intensity after OPA/3-MA derivatization was about 10-fold less than that of TRP (Table 2). However, it must be noted that the derivatized TRP was less stable than AMPH and *p*-OHA (Table 3). Variation in the stability

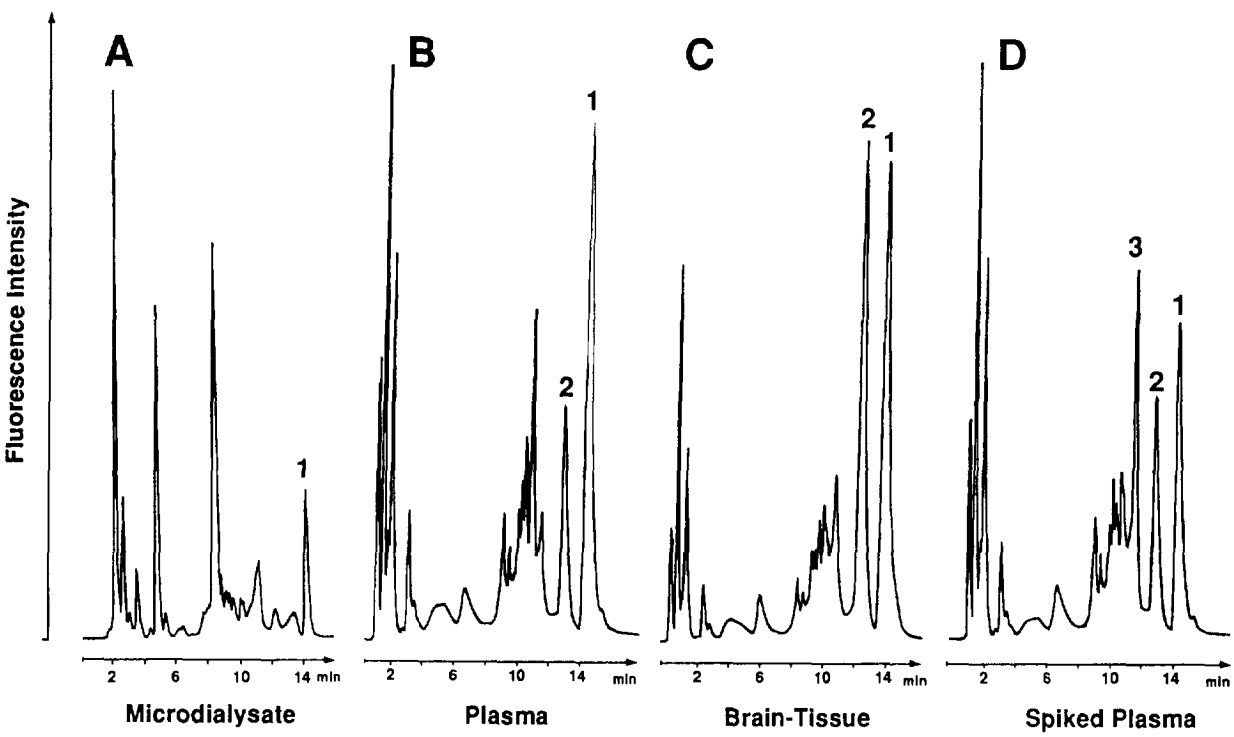

Fig. 1. Typical chromatograms showing the separation of OPA–3-MA derivatives of samples of microdialysate (A), plasma (B), and brain tissue (C) from a rat dosed subcutaneously with 5 mg/kg d-amphetamine sulfate, and of plasma (D) spiked with amphetamine, tryptamine, and *p*-hydroxyamphetamine. Peaks: 1 = d-amphetamine, 2 = tryptamine (internal standard), 3 = *p*-hydroxyamphetamine. Early peaks mainly represent unknown amounts of different amino acids contained in the sample. (A) 10 μl of brain microdialysate were diluted with 70 μl of H_2O and reacted with 20 μl of OPA–3-MA. (B) Blood plasma containing 0.8 μ*M* internal standard (theoretical injected concentration). (C) Brain tissue extract from the same animal (tryptamine, internal standard: 2.0 μ*M* concentration of the injected sample). (D) Plasma spiked with 5 μl of 20 μ*M* of each standard compound resulting in a theoretical 0.83 μ*M* concentration of the injected sample.

of different OPA–3-MA derivatives has been reported by others too [19].

3.3. Microdialysis samples

Peak AMPH concentrations in brain microdialysates (microdialysis probe implanted into the striatum) of six-month-old male Sprague–Dawley rats after one to three consecutive subcutaneous injections of 5 mg/kg d-AMPH sulfate (injected every 2 h) varied between 0.8 μM and 1.5 μM (i.e. 1.1 and 3.0 ng/10 μl, the concentration in the volume of the collected microdialysate sample). It should be noted that the in-vivo efficiency of microdialysis probes for AMPH is about 10%, i.e. true extracellular concentrations are ca. 10-fold higher than the concentration determined in the sample by HPLC. A typical chromatogram of such a microdialysis sample is shown in Fig. 1A, as compared to the chromatogram of a blank microdialysis sample (Fig. 2D), which still contains a number of other OPA–MA-derivatives, preferentially amino acids. Note that concentration and spectrum of amino acids in the microdialysate vary during the course of a brain microdialysis session, resulting in a variable peak-pattern of microdialysate chromatograms. It can be seen that AMPH is eluting later than all amino acids (Table 2). Standard curves for AMPH spiked into microdialysate buffer together with similar concentrations of TRP and *p*-OHA at increasing concentrations are depicted in Fig. 3. The regression fit was linear between 0.01 and 0.2 μM (which corresponds for AMPH to 1.35 and 27 ng/ml) with R^2s of 0.973 for AMPH, 0.876 for TRP and 0.964 for *p*-OHA. The detection limit (defined as twice the background noise) for microdialysate samples under the described conditions was 370 fmol (50 pg) with a minimum quantifiable limit of 740 fmol (100 pg). Intra- and inter-assay precision and

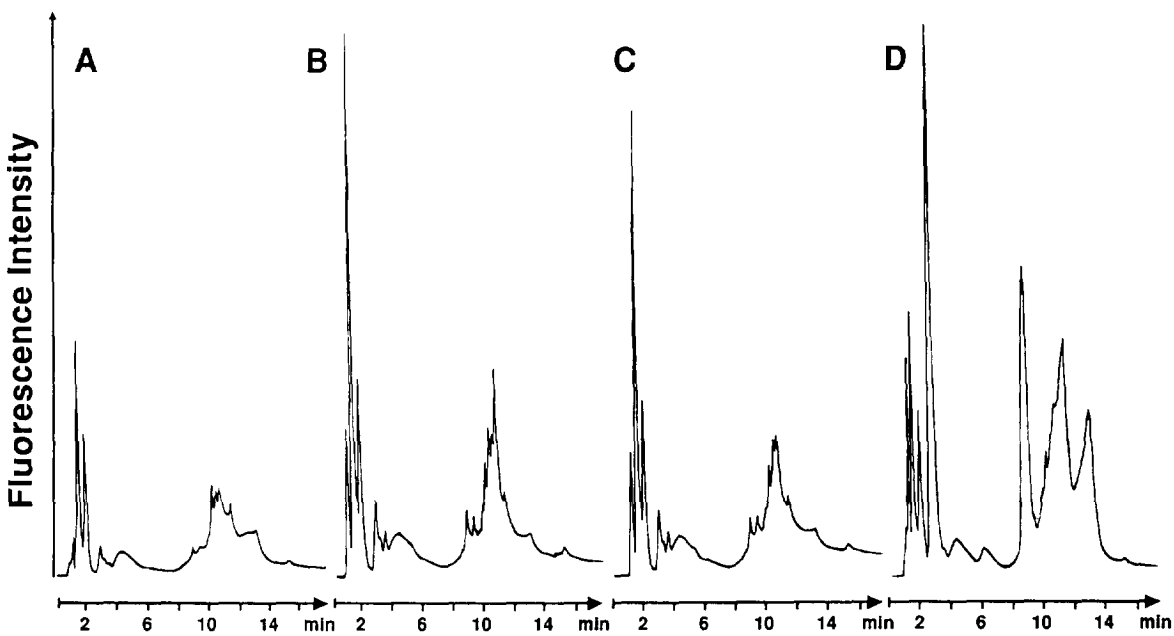

Fig. 2. Chromatograms of OPA–3-MA derivatives of microdialysate buffer only (A), either plasma (B) or brain (C) extract from a rat not dosed with amphetamine, or brain microdialysate collected before dosing the animal. Peaks represent unknown amounts of different amino acids and other biological amines contained in the sample. It should be noted that a surge in the release of most biological amines occurs after the introduction of the microdialysis probe producing the higher levels of "background noise" in panel D as compared to panel A.

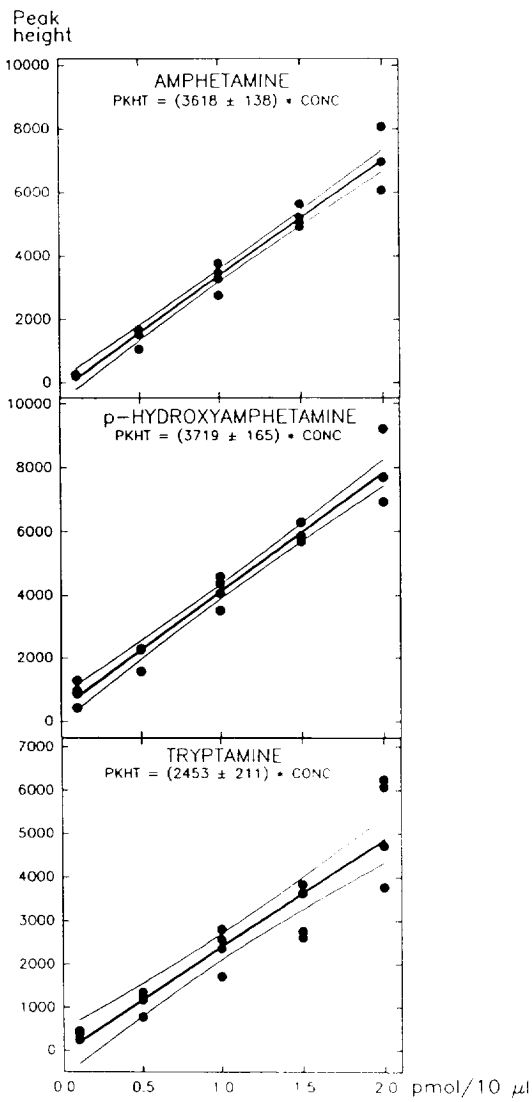

Fig. 3. Representative standard curves for d-amphetamine, *p*-hydroxyamphetamine, and tryptamine spiked into microdialysate buffer and derivatized with OPA–3-MA. For all three compounds the intercepts of the linear regression functions were not significantly different from zero. Therefore, only the regression coefficient (± S.E.M.) is depicted.

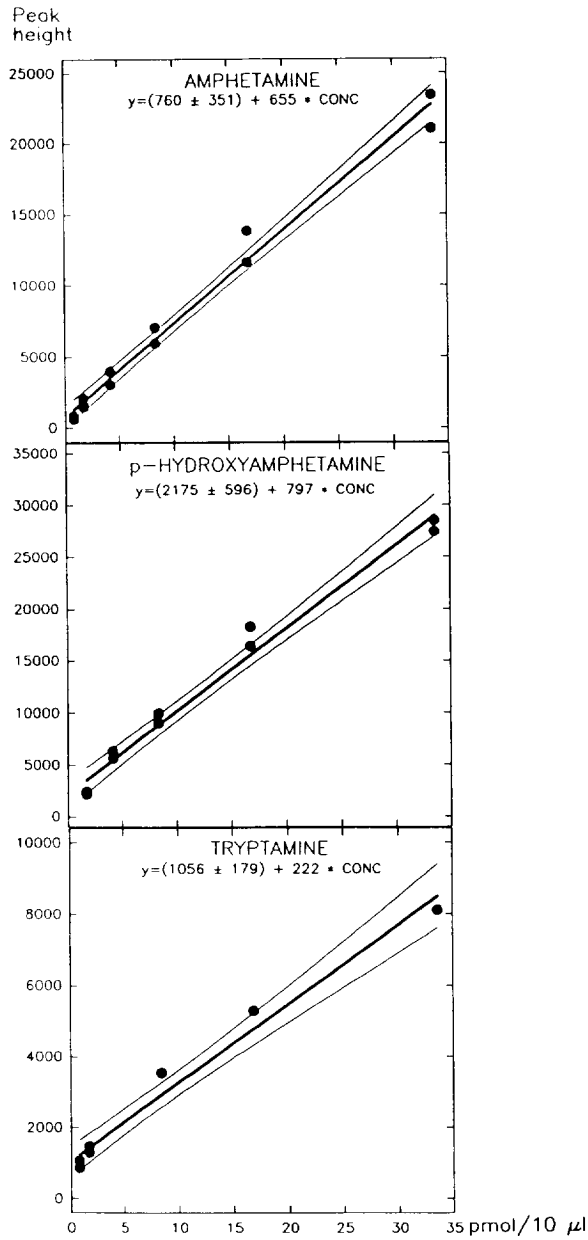

Fig. 4. Representative standard curves for d-amphetamine, *p*-hydroxyamphetamine, and tryptamine spiked into and extracted from plasma, subsequently derivatized with OPA–3-MA. Higher background noise for extracted tissue as compared to brain microdialysate resulted in intercepts of the regression functions significantly different from zero.

accuracy are demonstrated in Table 4. Intra-assay precision varied from 6.4 to 11.6%, and inter-assay precision varied from 3.2 to 16.2%. The average bias from the nominal concentration was 0.2–5.4% intra-assay, and 1.1–6.5% inter-assay.

Table 4
Intra- and inter-assay validation for amphetamine

Nominal concentration (ng/ml)	Intra-assay		Inter-assay	
	Mean ± S.E.M. (ng/ml)	R.S.D. (%)	Mean ± S.E.M. (ng/ml)	R.S.D. (%)
13.25	12.59 ± 0.38	5.6	12.39 ± 0.54	8.7
26.50	26.45 ± 1.69	6.4	24.49 ± 1.98	16.2
53.00	55.88 ± 3.25	11.6	53.39 ± 0.87	3.2

(Mean ± S.E.M. and R.S.D. of 4 determinations each)

3.4. Plasma and brain samples

The detection limit for extracted samples was 1.5 pmol (200 pg) of AMPH, and regression fits of extracted plasma standards are illustrated in Fig. 4. The regression fit was linear between 0.08 and 3.4 μM in the injected sample (which corresponds for AMPH to 11 and 460 ng/ml) with R^2s of 0.985 for AMPH, 0.976 for TRP and 0.980 for *p*-OHA. The percent recovery was assessed by comparing peak heights of extracted standards as compared to standards spiked into phosphate buffer (pH 2.6) with the addition of the appropriate volumes of borate buffer to create the required basic milieu for OPA derivatization. The percent recovery was linear between 0.4 and 3.3 μM (equivalent to 54 and 446 ng/ml) and was an average 86% for the first and 99% for the first plus second plasma extraction (Table 5), which is in accordance with data reported elsewhere [9]. For brain samples the percent recovery was an estimated 80% for the first and 95% first plus second extraction.

Rats exhibited average plasma concentrations of 1.6 μM (216 ng/ml) 40 min after the first and of 2.9 μM (392 ng/ml) 40 min after the third injection of 5 mg/kg d-AMPH sulfate. Although *p*-OHA was demonstrated for spiked plasma samples (Fig. 1D), it could not be detected in any of the samples from dosed animals. Concentrations in the striatum 40 min after the first and third injection of 5 mg/kg d-AMPH sulphate

Table 5
Extraction recovery of plasma samples spiked with amphetamine

Injected concentration	Recovery (%)		
μM (pg/μl)	Reference PKHT	1st Extraction PKHT	1st and 2nd Extraction PKHT
0.42 (56)	5130	4567 (89)	5305 (103)
0.83 (113)	10 296	8729 (85)	9789 (95)
1.67 (226)	20 504	16 667 (81)	19 116 (93)
2.50 (338)	31 559	27 659 (88)	33 370 (106)
3.33 (450)	39 932	35 854 (90)	39 133 (98)

Plasma (45 μl) was spiked with 5 μl of standard solution (10, 20, 40, 60, 80 μM of amphetamine). The dried extract was re-dissolved in 80 μl of KH_2PO_4; 20 μl of borate and 20 μl of OPA–3-MA were added. The reference standard was produced by mixing 5 μl standard solution with 75 μl of KH_2PO_4, 20 μl of borate and 20 μl of OPA–3-MA. Values represent averages of $n = 4$ for each concentration. Peak heights (PKHT) were corrected for the percent solvent volume used during the extraction procedure (see method description).

varied from 18 μM to 96 μM (2.4 to 13 μg/ml). Fig. 1 demonstrates typical chromatograms of extracted plasma and brain samples as compared to extracted blanks (Fig. 2).

3.5. Evaluation of the method

Farrell and Jefferies [9] compared five different HPLC methods for determining AMPH in urine and plasma, and those three that employed fluorometric detection were more sensitive than the UV methods. However derivatization with OPA–mercaptoethanol, although praised as technically most simple, was least sensitive and the derivatization products were less stable than those from derivatization with β-naphthaquinone-4-sulphonate (NQS) or 4-chloro-7-nitrobenz-2,1,3-oxadiazole (NBD-Cl). Derivatization with OPA–3-MA as presented here keeps the benefits of the OPA–mercaptoethanol procedure in being simple, fast and (in contrast to NBD-Cl) non-hazardous. In addition, it has a 4-fold greater sensitivity than that reported for the NQS-method described by Farrell and Jefferies [22]. To our knowledge only one other HPLC-method has been published, which directly addressed the determination of AMPH exceeding the sensitivity of the present procedure [25]. However this method (detection limit: 50 fmol AMPH) requires a chemiluminescence detector, and, under the described chromatographic conditions, AMPH elutes only after 65 min.

Another advantage of using 3-MA as a component in the OPA reagent is the high stability of the derivatives (Table 3), which enables samples to be run several hours after derivatization, allowing for overnight analysis with the use of an autosampler. With 2-mercaptoethanol, the "principal" reducing OPA-reagent, derivatized AMPH was found to be stable only for 60 min, thereafter decreasing to a lower level [22].

In conclusion, HPLC determination of AMPH with fluorometric detection after derivatization with OPA and 3-MA is a very simple, sensitive and rapid method and compares more favorably in these respects than most of the HPLC-fluorometric methods reported before. Investigators already using OPA derivatization and fluorometric detection to determine amino acids should be able to rapidly implement this method.

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