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Note

Sensitive method for the high-performance liquid chromatographic determination of amphetamine in urinary extracts

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High-performance liquid chromatography (HPLC) has up to now not been applied to the determination of amphetamine and related drugs in urinary extracts. However, other biogenic primary amines have been determined by HPLC after precolumn fluorescence derivatization with *o*-phthaldialdehyde reagent (OPT)[1–5]. The isoindole derivatives formed are highly fluorescent. A very sensitive analysis of amino acids was reported by Lindroth and Mopper[6]. Unfortunately the OPT derivatives were not stable in aqueous solution. Hence, the reaction time had to be carefully controlled before injection of the sample into the liquid chromatograph. Our initial study of the OPT derivatives of the two primary amines amphetamine and aniline showed maximum fluorescence intensity when the compounds were incubated with OPT for 5 min at room temperature prior to chromatographic analysis. A classical extraction technique for recovery of amphetamine from urine eliminated interfering biogenic amines and amino acids. This extraction combined with a precolumn fluorescence derivatization with OPT made possible a sensitive HPLC determination of amphetamine in urine.

EXPERIMENTAL

Apparatus

A Perkin-Elmer Series 1 reciprocating pump and an MPF 2A spectrophotofluorimeter equipped with a micro flow-cell (cell volume, 20 μl) were used. The fluorescence intensity was monitored at the emission wavelength of 450 nm with the excitation wavelength set at 350 nm. The chromatographic column was 10 cm × 0.46 mm I.D. stainless-steel tubing packed with RP-18 (10 μm; Brownlee Labs., Santa Clara, CA, U.S.A.). The mobile phase was methanol–water (75:25) with a flow-rate of 0.8 ml/min. The liquid chromatograph was connected to a Sigma 10 chromatography data station (Perkin-Elmer).

Reagents

The solvents methanol and diethyl ether were of HPLC grade (J. T. Baker, Phillipsburg, NJ, U.S.A.). The internal standard solution was prepared by dissolving *ca.* 40 mg aniline in 50 ml of deionized water. The derivatization reagent was prepared according to Lindroth and Mopper[6]. A urine drug standard was prepared by adding amphetamine (1.0 μg/ml as free base) to a drug-free urine.

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Procedure

To 500 μl urine were added 10 μl of the internal standard solution and 100 μl of 1 *M* NaOH. After thorough mixing, 1 ml diethyl ether was added and the tube was shaken in a vortexer for 1 min. After centrifugation ($\geqslant$ 1000 *g*) for 5 min, the diethyl ether layer was transferred to a conically tipped tube with the aid of a pasteur pipette. Then 50 μl of 0.1 *M* HCl were added and the tube was vortexed for 1 min and briefly centrifuged. The diethyl ether layer was aspirated off and discarded. To 25 μl of the HCl extract were added 2.5 μl of 1 *M* NaOH. The tube was briefly vortexed and 100 μl of the OPT reagent were added. After further vortexing, the mixture was incubated at room temperature for exactly 5 min. Then 15 μl of the solution were immediately injected in the liquid chromatograph.

RESULTS AND DISCUSSION

The primary amines detected with this procedure (see Table II) are resolved under the chromatographic conditions used. Fig. 1 shows both a urine blank

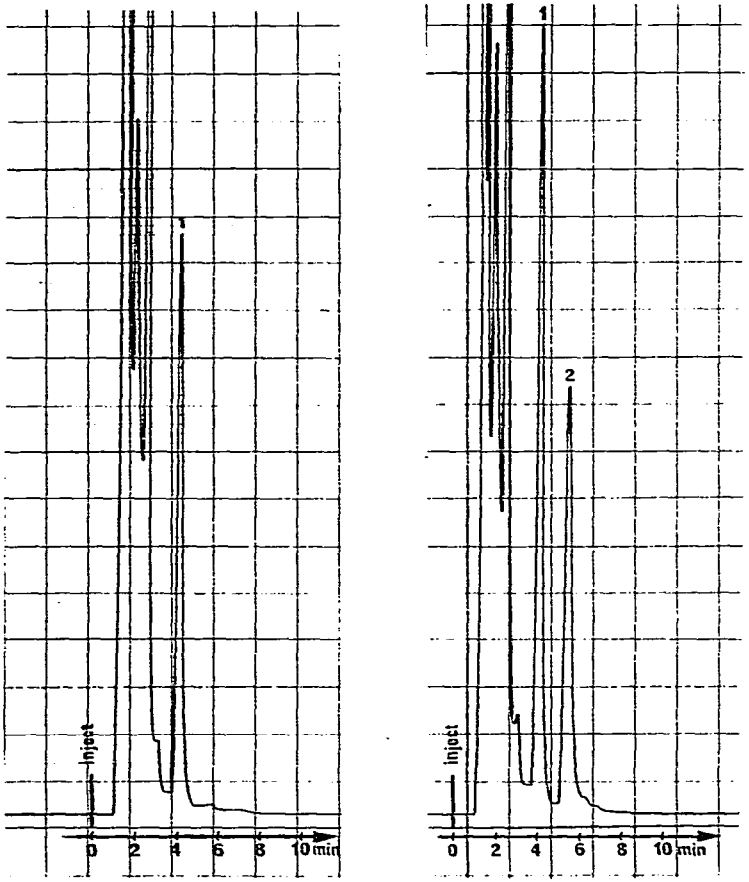

Fig. 1. Chromatograms of (left) an analysis of a urine blank with added internal standard and of (right) a urine amphetamine standard run through the procedure (0.5 μg/ml, as free base). Peaks: 1 = aniline; 2 = amphetamine. Retention times are given in Table II.

TABLE I

PRECISION

CV = coefficient of variation.

Level 1 (0.45 μg/ml)			*Level 2 (2.50 μg/ml)*		
n	*Mean (μg/ml)*	*CV*	*n*	*Mean (μg/ml)*	*CV*
10	0.40	8.8	10	2.50	4.1

and an amphetamine standard run through the procedure. Fifty blank urines were analyzed and typical amphetamine blank values were all in the range 0–0.08 μg/ml as computed by the Sigma 10 data station. In one of the samples a peak of unknown identity and having a retention time of 6.3 min was observed.

No peaks interfering with that of aniline were detected in chromatograms obtained from several samples. The OPT derivatives had a tendency to give memory peaks in the liquid chromatographic system used. This effect was eliminated by repeated injections of 15 μl of OPT reagent immediately after a completed analysis of an amphetime-containing sample (unknown or standard).

A practical detection limit for amphetamine was set at 0.2 μl/ml. The calibration graph of peak area ratio of the drug and internal standard *versus* amphetamine concentration was linear in the range 0.25–3.0 μg/ml. The correlation coefficient was 0.96. Table I gives precision data obtained by repeated analysis of a urine to which amphetamine had been added.

No interference to amphetamine was found from the primary amines norephedrine, phentermine and chlorophentermine, or from the drugs sulfametizole, salicylamide, salicylic acid, acetaminophen, phenazone, primidone, ethosuximide, phenytoin, carbamazepine, meprobamate, tybamate, hexapropymate, phenaglycodol, methaqualone, diphenhydramine, diazepam, desmethyldiazepam, oxazepam, phenobarbital, heptabarbital, bupivacain, lidocain, ephedrine, diethylpropione, methylphenidate, phenmetraline, methadone, nicotine, caffein, theophylline, amitriptyline, nortriptyline, desipramine, imipramine and trimipramine. Although phentermine and chlorophentermine were detected with this procedure the sensitivity for these two compounds (detection limit 1.5 μg/ml) was considerably lower than that for amphetamine.

TABLE II

RETENTION TIMES OF STIMULANTS AS OPT DERIVATIVES

Norephedrine is eluted early with the "OPT-solvent peaks".

Peak no.	*OPT derivative of*	*Retention time (min)*
1	Aniline	4.4
2	Amphetamine	5.5
	Phentermine	6.8
	Chlorophentermine	10.0

No metabolites were studied. This method was used to verify the presence of amphetamine, and the high concentrations of phentermine and chlorophentermine in urines already screened by a previously reported method[7].

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