Journal of Chromatography, 284 (1984) 457–461
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,343

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR POST-COLUMN, IN-LINE DERIVATIZATION WITH *o*-PHTHALALDEHYDE AND FLUOROMETRIC DETECTION OF PHENYLPROPANOLAMINE IN HUMAN URINE

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(First received July 25th, 1983; revised manuscript received October 4th, 1983)

SUMMARY

A rapid and sensitive method for the analysis of phenylpropanolamine in urine was developed using high-pressure liquid chromatography, post-column, in-line derivatization with *o*-phthalaldehyde, followed by detection with a fluorometer. Human urine was injected directly into the chromatographic system, and phenylpropanolamine separated in the reversed-phase mode.

The practical lower limit of detection was 0.1 μg/ml of urine. The coefficient of variation from 0.96 to 96 μg/ml varied between 2.23 and 0.19%, respectively. The linearity of the calibration graphs is excellent ($r = 0.9999$) over a concentration range of two orders of magnitude.

INTRODUCTION

Phenylpropanolamine, a widely used sympathomimetic agent, and related phenylalkanolamines have been assayed in biological fluids by gas–liquid chromatography (GLC) following extraction and conversion to electron-capturing perfluoroacyl or pentafluorobenzylimine-trimethylsilyl derivatives[1–10]; GLC following formation of a pentafluorophenyl-oxazolidine derivative and extraction[11]; GLC following extraction using a nitrogen selective detector[12]; thin-layer chromatography (TLC) following acetylation with tritiated acetic anhydride[13]; and high-performance liquid chromatography (HPLC) following extraction and pre-column derivatization with *o*-phthalaldehyde[14], 4-chloro-7-nitrobenz-2,1,3-oxadiazole and sodium β-naphthaquinone-4-sulphonate[15], and phenylisothiocyanate[16]. All these methods require extraction steps, and most require derivatization.

Preliminary studies showed that HPLC with absorbance detection lacked the necessary sensitivity and specificity. *o*-Phthalaldehyde in the presence of 2-mercaptoethanol has been shown to react with primary amines to form fluorescent prod-

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ucts[17–20]. The reaction proceeds rapidly at room temperature, forming fairly stable products.

A sensitive, rapid method for separation of phenylpropanolamine and an internal standard by HPLC followed by post-column in-line derivatization with *o*-phthalaldehyde and fluorometric detection is presented in this report. The method can detect as little as 0.1 μg/ml in urine and can quantitate urine concentrations above 1 μg/ml with accuracy and precision suitable for pharmacokinetic studies. Sample preparation is a simple, rapid process that allows analysis of large numbers of samples quickly.

EXPERIMENTAL

Apparatus

The liquid chromatographic system was assembled from two Waters Assoc. (Milford, MA, U.S.A.) Model 6000 A solvent delivery systems; a Waters Assoc. Model 710 B WISP automatic sample processor, a 12.5 cm × 4.6 mm I.D. stainless-steel column packed with RP-18 (5 μm ODS-Hypersil; Shandon Southern Instruments, Sewickley, PA, U.S.A.), a 3 cm × 4.6 mm I.D. stainless-steel guard column packed with RP-18 (10 μm ODS LiChrosorb; Brownlee Labs., Santa Clara, CA, U.S.A.), a Schoeffel Instrument Corp. (Westwood, NJ, U.S.A.) Model FS 970 Spectrofluorometer operated at λ_{ex} = 340 nm and λ_{em} = 418 nm; coiled polytef tubing (4.8 m × 0.7 mm I.D.); which served as a post-column, in-line reactor, and an Altech (Arlington Heights, IL, U.S.A.) T-fitting, which served to connect the reactor, one pump, and column. The liquid chromatograph was connected to a Spectra-Physics (Santa Clara, CA, U.S.A.) Model 4100 integrator–calculator.

Reagents

Phenylpropanolamine hydrochloride and amphetamine sulfate were obtained from Sigma (St. Louis, MO, U.S.A.). The solvent methanol was analytical-reagent grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Reagent grade 2-mercaptoethanol and *o*-phthalaldehyde were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.) and Sequanol grade triethylamine was obtained from Pierce (Rockford, IL, U.S.A.). 1-*n*-Hexanesulfonate as the sodium salt was obtained from Regis Chemical (Morton Grove, IL, U.S.A.).

Solutions

The mobile phase was composed of 0.05 *M* sodium dihydrogen phosphate, 0.01 *M* 1-*n*-hexanesulfonate sodium salt, 0.0072 *M* triethylamine adjusted to pH 3.0 with phosphoric acid and 40% methanol. The volumetric flow-rate of the mobile phase was 1.5 ml/min.

The *o*-phthalaldehyde solution introduced into the mobile phase, post-column, was prepared as follows: *o*-phthalaldehyde (800 mg) was dissolved in methanol (10 ml) followed by addition of 2-mercaptoethanol (2 ml) and enough 0.4 *M* borate buffer (pH 10.4) to make 1 l of solution. Volumetric flow-rate of this solution was 1.5 ml/min.

Stock solutions of phenylpropanolamine and internal standard amphetamine (1 mg/ml in water) were prepared and stored at 4°C. A standard curve was generated

by spiking blank urine samples (2 ml) with varying amounts of phenylpropanolamine and a constant amount of internal standard. Microliter aliquots of the stock solution of phenylpropanolamine and amphetamine stock solution were added to urine. The final internal standard concentration in urine was 20 μg/ml. The phenylpropanolamine concentration range in urine was 0.96–96 μg/ml. These samples were treated according to the following analysis procedure.

Analytical procedure

Internal standard (amphetamine, 20 μg/ml urine) was added to urine samples (2 ml) in 15-ml glass-stoppered centrifuge tubes. After vortex mixing followed by centrifugation at $\geqslant 1000\ g$ for 5 min, the supernatant was transferred to autosampler vials for injection into the high-performance liquid chromatograph and 25-μl aliquots were injected.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatograph for phenylpropanolamine in spiked human urine and in urine obtained from a human subject after oral ingestion of 25

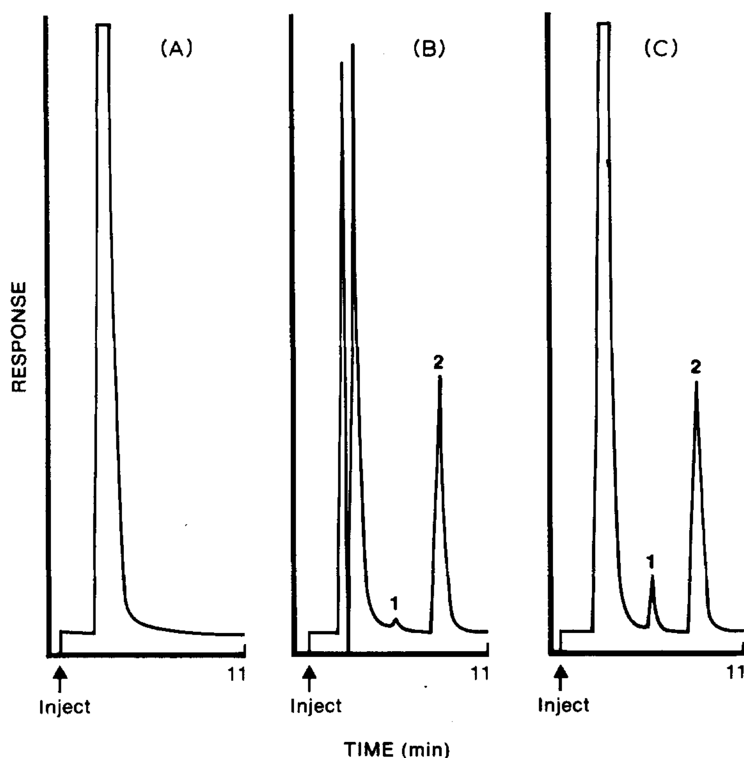

Fig. 1. Chromatograms of an analysis of a urine blank (A); a urine sample with added phenylpropanolamine and internal standard (amphetamine) (B) 2.96 and 20 μg/ml, respectively; a urine sample from a subject dosed with 25 mg phenylpropanolamine hydrochloride with added internal standard (C) (13.96 and 20 μg/ml, respectively). Peaks: 1 = phenylpropanolamine, retention time = 5.4 min; 2 = internal standard amphetamine, retention time = 8.0 min.

mg phenylpropanolamine hydrochloride. Analysis of predose urine samples from 24 human subjects presented no chromatographic peaks that would interfere with either phenylpropanolamine or the internal standard. Plots of peak area ratios (phenylpropanolamine to internal standard) against phenylpropanolamine concentrations were linear. Typical standard curves for phenylpropanolamine in urine had correlation coefficients of 0.9999.

The coefficients of variation for five replicate assays at 0.96, 3.82, 9.55, 38.2, and 96 μg/ml of urine were ± 2.23, ± 0.55, ± 0.41, ± 0.64, and ± 0.19% respectively. The lowest standard routinely used was 0.96 μg/ml; however, the achievable detection limit of the method described is 0.1 μg/ml of urine. The high sensitivity of the method is due to the specificity of the fluorescence detection step for phenylpropanolamine relative to the low biological background. The sample preparation procedure is rapid, allowing analysis of up to 100 samples per day.

To demonstrate the usefulness of this method, results from analysis of urine samples obtained from three healthy subjects who had ingested 25 mg phenylpropanolamine hydrochloride in water are shown in Table I. Urinary recovery from these three subjects was 80.4, 76.5, and 76.5% respectively. Earlier reports[21–23] suggest that very little metabolism of phenylpropanolamine occurs. One investigator reported that 90% of the 25 mg oral dose was excreted in the urine as unchanged phenylpropanolamine[21,22], while in another report[23] 80–90% of the dose was excreted unchanged within 24 h. This agrees with recoveries seen in the three subjects above, as well as those seen in subsequent studies with other subjects.

TABLE I

URINARY EXCRETION OF PHENYLPROPANOLAMINE IN HUMANS AFTER A SINGLE 25 mg ORAL DOSE OF PHENYLPROPANOLAMINE HYDROCHLORIDE

Hours	*Urinary phenylpropanolamine (mg as free base)*		
	Subject 1	*Subject 2*	*Subject 3*
0–4	6.2	7.9	6.4
4–8	5.0	3.4	4.9
8–12	1.6	2.2	1.4
12–16	1.8	0.7	2.1
16–24	0.9	1.2	0.6
24–48	0.7	–	–
48–72	–*	–	–
72–96	–	–	–

* No phenylpropanolamine detected.

No interferences to either phenylpropanolamine or amphetamine were found from the following compounds which are primary amines detected and resolved by the method: phenylethylamine, norepinephrine, dopamine, nordefrin, metaraminol, tyramine, hydroxyamphetamine, methoxamine, phentermine and tuaminoheptane. Secondary and tertiary amines are not detected by this method, therefore no responses were seen from the following compounds when tested: epinephrine, salbutamol, terbutaline, phenylephrine, metanephrine and ephedrine.

Although the method was developed with the purpose of studying the pharmacokinetics of phenylpropanolamine in humans following oral administration of the drug, it was also found to be suitable for analysis of phentermine, amphetamine, methoxamine, phenylethylamine and tuaminoheptane. 3-Aminopentane would be a suitable internal standard in the analysis of these compounds, as well as for phenylpropanolamine.

The method has been used to analyze phenylpropanolamine in over 2000 urine samples. The guard column was changed periodically during this time. No deterioration of the analytical column performance or of the assay as a whole was observed.

ACKNOWLEDGEMENTS

We thank Dr. Pieter Bonsen for critically reviewing this manuscript and Ms. Rose Wright for her extensive literature research.

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