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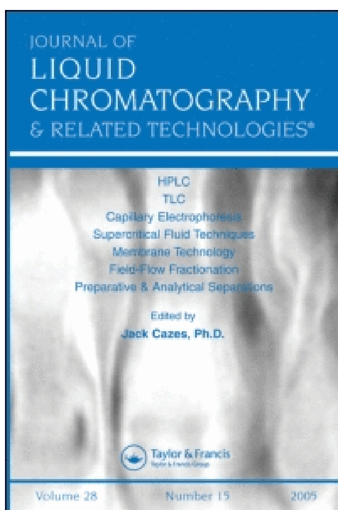


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# DETERMINATION OF PLASMA PSEUDO-EPHEDRINE BY FLUORESCENCE DETECTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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## ABSTRACT

A sensitive and specific high-performance liquid chromatographic method has been developed for the determination of pseudoephedrine in plasma. The assay was based on the production of a highly fluorescent derivative of pseudoephedrine using 4-chloro-7-nitrobenzo-2,1,3-oxadiazole (NBD-Cl) as the derivatization agent. The fluorescent derivatives were separated using normal phase liquid chromatography after an automated, column-switching, sample clean-up procedure. Pseudoephedrine determination was accurate and precise at concentrations as low as 10 nanograms per milliliter of plasma. The chromatographic step separated derivatives of pseudoephedrine from those of norpseudoephedrine and several other amines. Measurement of pseudoephedrine concentrations in plasma following a single 120 milligram oral dose is illustrated.



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## INTRODUCTION

Bioavailability and bioequivalence studies on pseudoephedrine formulations require highly sensitive methods for determination of the drug in biological materials. Gas chromatographic procedures have been described for analysis of plasma pseudoephedrine (1) or its heptafluorobutyl (2,3,) and trifluoroacetic anhydride (4,5,) derivatives. However, these methods are limited to plasma pseudoephedrine levels at or above the 40-50 ng/ml range. Lin, et. al. (6) reported a gas chromatographic method which was capable of quantifying pseudoephedrine at a plasma concentration of 15 nanograms/ml; however the method was very time consuming.

Very little information is available on high performance liquid chromatographic (HPLC) analysis of pseudoephedrine. An HPLC procedure for urinary pseudoephedrine was described by Lai, et. al. (7) which was specific and reproducible but too insensitive (1.5 μg/ml) for analysis of plasma levels. The present report describes a new HPLC procedure for plasma pseudoephedrine determination which achieves greater sensitivity (10 ng/ml) than the gas chromatographic methods by measuring a highly fluorescent 4-chloro-7-nitrobenzo-2,1,3,-oxadiazole derivative. Derivatization of various primary and secondary amines, and the use of the assay for analysis of pseudoephedrine in human plasma are illustrated.

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## MATERIALS AND METHODS

### Chemicals

Toluene, cyclohexane, methanol, butanol, ethyl ether (anhydrous) and methylisobutylketone were reagent grade and obtained from Mallinckrodt, Paris, Kentucky. The 4-chloro-7-nitrobenzo-2, 1, 3-oxadiazole (NBD-Cl) was obtained from Regis Chemical Co., Morton Grove, Illinois. Pseudoephedrine sulfate, ephedrine sulfate, phenylethylamine hydrochloride, phenylethanolamine, α-phenyl-glycinol (2-amino-2-phenylethanol), aspirin, acetaminophen, salicylamide, theophylline, and caffeine were obtained from Sigma Chemical Co., Saint Louis, Missouri. Chlorpheniramine, brompheniramine and their desmethyl derivatives were obtained from Schering Corporation, Bloomfield, N.J.

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### Standard Solutions

Stock solutions of pseudoephedrine sulfate and α-phenylglycinol (internal standard, Fig. 1) were prepared in distilled water at 1.0 mg base equivalent per ml. Aliquots were stored frozen until utilized to prepare working solutions of lower concentration, also diluted in water.

### Extraction and Derivatization Procedures

Internal standard (0.1 to 1.0 μg) was added to 1.0 ml of control human plasma containing various amounts of pseudoephedrine and the samples made alkaline with 0.5 ml of 25% $K_2CO_3$. The samples were shaken with 10.0 ml diethylether for 10

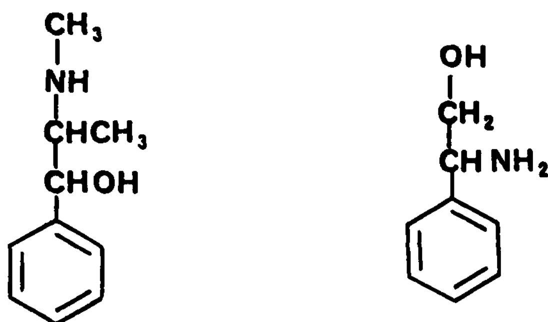


Figure 1. Structures of pseudoephedrine and internal standard (alpha-phenylglycinol)

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minutes and centrifuged for 5 minutes at 1000xg. The tubes were placed in a methanol-dry ice mixture until the aqueous layer was frozen. The ether layer was transferred to a separate tube and 1.0 ml of 0.05 N HCl was then added to the ether. The tubes were shaken for 5 minutes and centrifuged for 2 minutes at 1000xg. The aqueous layer was frozen by placing the tubes in methanol-dry ice, the ether layer was discarded and residual ether was removed by placing the tubes under a stream of $N_2$ after thawing.

The aqueous extract was adjusted to pH 8.5 by the addition of 40 µl NaOH (1.0N) and 0.5 ml borate buffer (0.2M, pH 8.5). NBD-Cl was then added (0.4 ml, 0.5% W/V in methylisobutylketone) and each tube heated at 79$^{o}$C for 1 hour in a multi-temp-block

(Lab-Line Instruments, Inc., Melrose Park, Ill.), mixing briefly at 5 minute intervals. The samples were cooled on ice, 3.0 ml cyclohexane was added, and each tube mixed on a vortex. After centrifugation at 1000 x g for 3 minutes, aliquots of the cyclohexane layer were injected into an HPLC system using a Waters Intelligent Sample Processor (Model 710A, Waters Associates, Millford, Mass.). If samples were not chromatographed on the day of extraction, the cyclohexane extract was kept overnight in the refrigerator (4$^{o}$C).

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## Chromatographic Conditions

An automated column-switching HPLC technique was used to determine derivatized pseudoephedrine concentration in plasma extracts. The system contained two HPLC columns. The first column was used to extract derivatized pseudoephedrine and internal standard from the reaction medium and a second column was used to determine pseudoephedrine quantity in each sample. A schematic of the HPLC system is presented in Figure 2. Pump A delivered cyclohexane and pump B delivered a mobile phase containing methanol:butanol:toluene:cyclohexane (in a ratio of 1:2:47:50). The switching valve allowed the mobile phases to take one of two paths illustrated by solid and dashed lines. In the first position (solvent flow shown by solid line), injected reaction medium was directed onto the extraction column where pseudoephedrine and internal standard were retained while unwanted components passed into the waste.

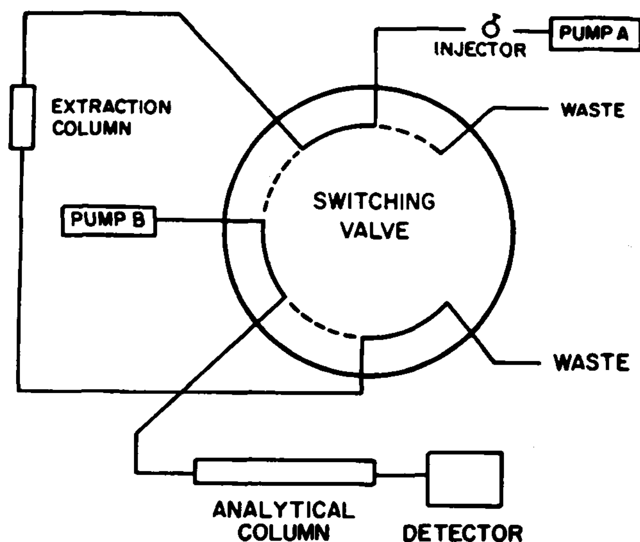


Figure 2. Schematic of the HPLC system

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Three minutes after injection the switching valve was automatically rotated to the second position (solvent flow shown by dashed lines). This placed the first column in series before the second column, allowing the second solvent to elute derivatized pseudoephedrine and internal standard onto the second column for detection and quantitation.

After sufficient time (2 minutes) had elapsed to ensure pseudoephedrine elution from the first column, the valve was automatically switched back to the first position, ready for the next injection. Mobile phases were pumped through the second column (Alltech Silica, 4.6 x 250mm) and through the first column

(Waters Guard Pak, CN) using Model 6000A solvent delivery systems (Waters Associates). Pumping rate was 2.0 ml/min. Effluent from the second column was monitored by an LDC Fluoro Monitor Model 1311 (Milton Roy Co., Riviera Beach, Florida) equipped with an F4T5-D lamp (General Electric), Wratten No. 47B filter on the primary excitation side (460 - 500 nM band-pass) and a Wratten No. 12 filter on the secondary emission side (500 nM cut-off). Sensitivity was set at 1.0 x 10 mV. All HPLC analyses were carried out at ambient temperature.

Fluorescence intensity was recorded on a Goerz Metrawatt SE 120 recorder and a Hewlett Packard Model 3357 laboratory data system. The HPLC system contained a 10 port helical air actuated valve (Valco Ins. Co., Houston, Texas, Model AC10µ). Valve switching times were controlled by a Control model CD timer; countdown was initiated by the auto-injector.

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Calculations

Chromatogram peaks were identified on the basis of retention times and calculations of pseudoephedrine concentration were based on the ratio of peak height of pseudoephedrine to that of the internal standard.

## RESULTS

The HPLC retention times for derivatives of pseudoephedrine, its metabolite (norpseudoephedrine), internal standard and other amines are listed in Table 1. Pseudoephedrine was well separated

Table 1

Retention Times for NBD-CL Derivatives of Pseudoephedrine, its Analogs, and other Compounds of Interest

| Compound | Retention Time (Min) |
|---|---|
| Phenylethylamine | 3.1 |
| Dimethoxyphenethylamine | 6.4 |
| Phenylpropanolamine | 6.4 |
| Ephedrine | 7.8 |
| Norpseudoephedrine | 7.9 |
| Phenylethanolamine | 8.8 |
| α-Phenylglycinol (internal standard) | 9.7 |
| Pseudoephedrine | 12.0 |
| Tyramine | 13.9 |
| Phenylephrine | 21.5 |
| Aspirin | N.D.[a] |
| Acetaminophen | N.D. |
| Salicylamide | N.D. |
| Theophylline | N.D. |
| Caffeine | N.D. |

[a]N.D. = None detected.

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from internal standard and its metabolite (norpseudoephedrine). Aspirin, acetaminophen and salicylamide which may be coadministered with pseudoephedrine are not amines and did not produce fluorescent derivatives. Theophylline and caffeine also did not produce derivatives.

A standard curve for pseudoephedrine and internal standard extracted from human plasma is illustrated in Figure 3. The peak

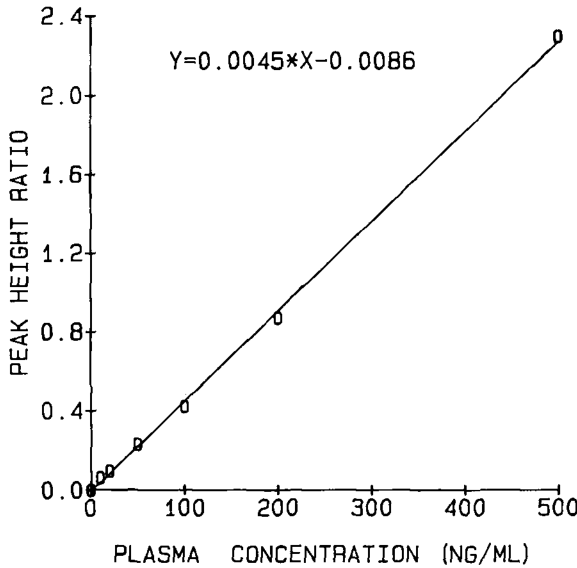


Figure 3. Concentration curve for pseudoephedrine extracted from human plasma. Internal standard concentration was 400 ng/ml plasma. Injection volume was 100 $\mu$l.

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height ratios (pseudoephedrine/internal standard) were linearly related to pseudoephedrine concentration over the range of 10 to 500 ng/ml with a regression coefficient ($r^2$) of 0.99. Subsequent testing has shown that the curve is linear up to at least 2000 ng/ml.

Accuracy and precision of the assay were demonstrated at several plasma concentrations as shown in Table 2. Over the range of 20 to 500 ng/ml the observed concentrations of pseudoephedrine reflected the excellent accuracy of the method

Table 2

Accuracy and Precision of Pseudoephedrine
Assay in Spiked Human Plasma

| Amount Added (ng) to 1 ml Plasma | Number of Replicates | Observed Concentrations (ng/ml) | C.V. % | Relative Error % |
|---|---|---|---|---|
| 500 | 6 | 506.6 | 2.1 | 1.3 |
| 200 | 8 | 191.2 | 8.6 | 4.4 |
| 100 | 5 | 91.7 | 4.8 | 8.3 |
| 50 | 8 | 50.2 | 10.6 | 4.0 |
| 20 | 8 | 20.4 | 7.0 | 2.0 |

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with the relative error falling in the range of 1.3 to 8.3% The precision was also good, with the percent coefficient of variation (% C.V.) in the range of 2.1 to 10.6.

The limit of quantitation for the assay was defined as the lowest concentration of pseudoephedrine where accuracy (relative error) and precision (coefficient of variation) did not exceed 20%. As shown in Table 3 these conditions hold true at a pseudoephedrine concentration of 10 ng per ml of plasma. Concentration values below this limit and above the limit of detection (approximately 2 ng/ml) were considered estimates. The chromatographic response to 10 ng of pseudoephedrine extracted from 1 ml human plasma and the corresponding response to blank plasma are shown in Figure 4.

An examination of pseudoephedrine stability in frozen plasma and a comparison of between-run and within-run precision is

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Table 3

Replicate Analysis of Pseudoephedrine Extracted from Plasma at the Lowest Level of Quantitation

| Pseudoephedrine Added (ng/ml) | Observed Concentration (ng/ml) |
|---|---|
| 10 | 10.47 |
| 10 | 11.27 |
| 10 | 10.09 |
| 10 | 10.34 |
| 10 | 10.03 |
| 10 | 10.34 |
| 10 | 13.39 |
| 10 | 11.13 |
| | 10.63 |
| Mean | 10.85 |
| C.V. | 9.6% |
| Relative Error | 8.5% |

Table 4

Between-Run and Within-Run Precision of Replicate Analysis

| Assay Date | Number of Samples | Mean | (C.V.) | Relative Error % |
|---|---|---|---|---|
| 12/4/85 | 5 | 215.1 | (8.4) | 7.5 |
| 12/17/85 | 5 | 190.4 | (5.9) | 4.8 |
| 1/8/86 | 5 | 188.2 | (2.7) | 5.9 |
| 1/27/86 | 5 | 193.8 | (1.1) | 3.1 |
| 3/10/86 | 7 | 200.9 | (3.8) | 0.4 |
| Between-run mean (C.V.) | | 197.7 | (5.5) | |

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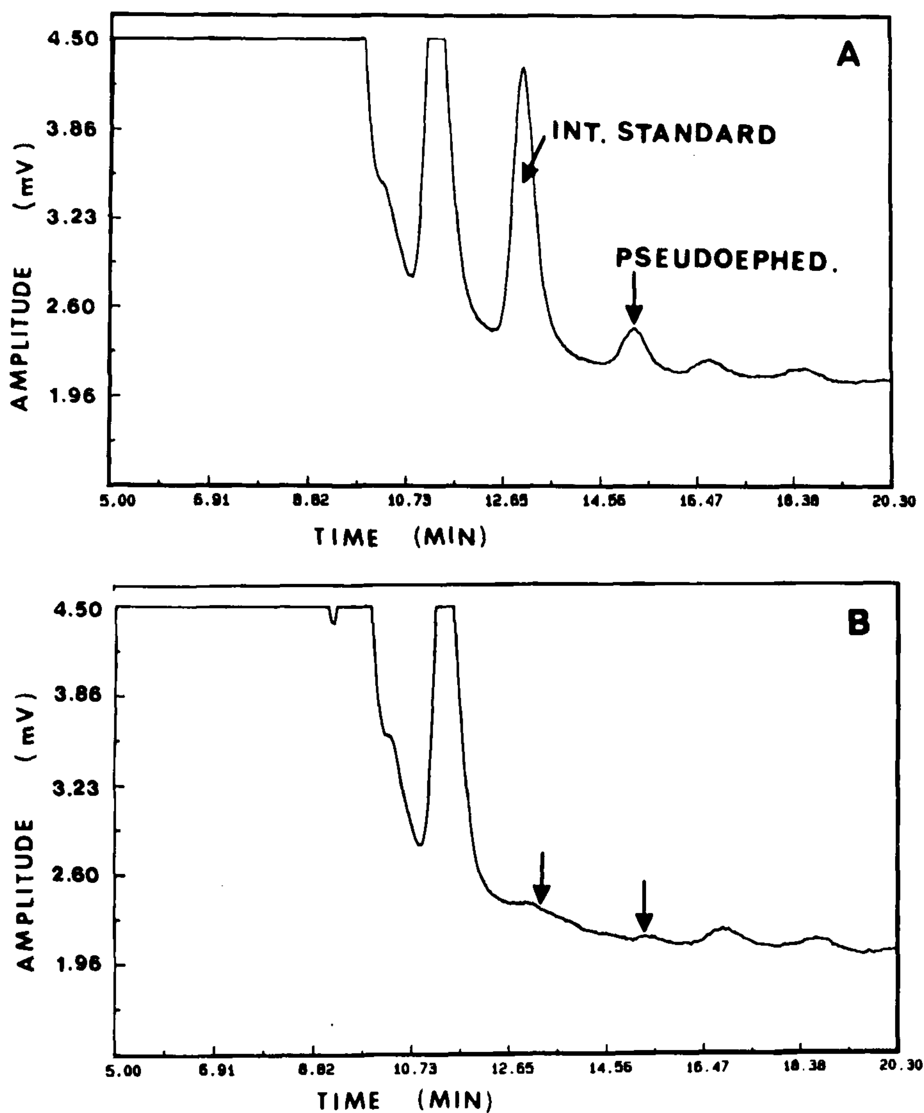


Figure 4. Chromatographic response to 10 nanograms pseudoephedrine and 100 nanograms internal standard extracted from human plasma (A), and background response to control human plasma (B). Injection volume was 200 $\mu$l.

presented in Table 4. For this study, a pool of human plasma containing pseudoephedrine (200 ng/ml) was assayed on the day of preparation and on subsequent days. The plasma was stored at -20$^{o}$C. As can be seen, the within-run and day-to-day accuracy and precisions were good. In addition, pseudoephedrine was stable for at least the 97 day duration of this experiment.

The method was used to examine pseudoephedrine plasma levels in volunteers receiving sustained-release formulation containing 120 mg of pseudoephedrine sulfate (Afrinol Repetabs, Schering). The data is presented in Figure 5. Each point is the mean of plasma levels found in 12 subjects. Mean values for AUC, Cmax and Tmax were 3686 hr x ng/ml, 262 ng/ml, and 6.5 hr, respectively.

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## DISCUSSION

The derivatization of pseudoephedrine with NBD-Cl resulted in a highly fluorescent product which facilitated the development of a sensitive and specific analytical method. NBD-Cl was first used for the fluorogenic labelling of amines and amino acids by Gosh and Whitehouse (8) and applied to the analysis of methylcarbamate insecticides by Lawrence and Frei (9). Ahnoff, et. al (10) conducted a detailed study of the derivatization reaction and reported reverse-phase HPLC chromatographic systems for separating several amino acids derivatized with NBD-Cl. However, the NBD-amine derivatives display very weak fluorescence

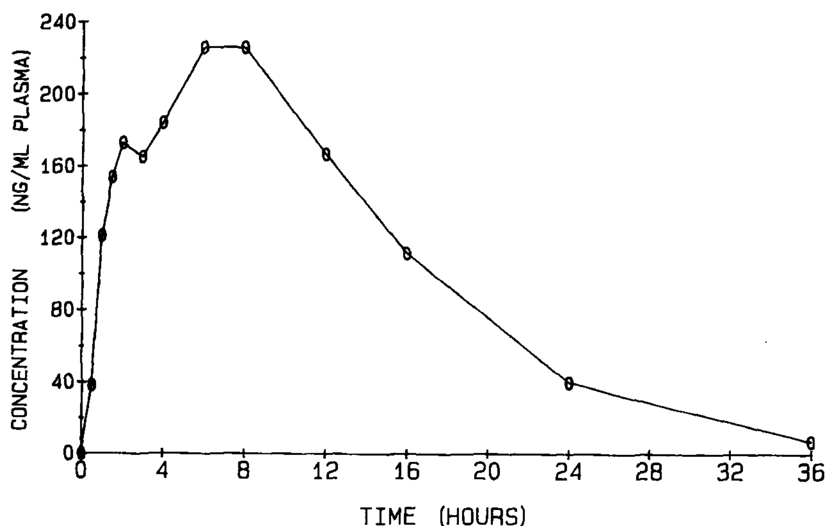


**Figure 5. Mean plasma levels of pseudoephedrine in twelve normal subjects following a 120 mg oral dose of pseudoephedrine sulfate in a repeat action tablet.**

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in polar solvents but are strongly fluorescent in non-polar organic solvents (9). Therefore we carried out the HPLC separation in normal phase mode, as had been done by Wolfram, et. al. (11), on a 10 micron silica column.

Both primary and secondary amines may be derivatized as shown in Table 1. The data also show that several compounds such as aspirin or caffeine, which may be present in the plasma sample, did not produce fluorescent derivatives and would not interfere with pseudoephedrine determination. These results indicate that the HPLC method determines pseudoephedrine

specifically, without interference from its metabolite, its analogs and other common drugs.

The sensitivity was sufficient for detailed pharmacokinetic studies requiring measurement of pseudoephedrine at long time intervals after dosing. The data presented in Figure 5 and the resulting mean values for AUC, Cmax and Tmax were in agreement with those published by Lin, et. al. (12,13) in which subjects also received 120 mg of pseudoephedrine sulfate in a repeat action tablet. Similar AUC and Cmax values were reported by Graves, et. al. (5) in subjects receiving a single 120 mg dose of pseudoephedrine hydrochloride in a liquid controlled-release product.

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We have found the method to be very reproducible, accurate, sensitive and specific for pseudoephedrine. As we have demonstrated, it is useful in pharmacokinetic studies and has been used routinely in our laboratory to determine pseudoephedrine plasma levels in bioequivalency studies. In addition, the procedure can be modified for the assay of many amines such as ephedrine, phenylpropanolamine and norpseudoephedrine.

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