Journal of Chromatography, 310 (1984) 412—417
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2218

Note

Determination of fenfluramine and norfenfluramine in plasma using a nitrogen-sensitive detector

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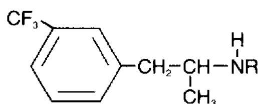
(First received February 16th, 1984; revised manuscript received May 17th, 1984)

Fenfluramine (I, Fig. 1) is currently used as an appetite suppressant and is used experimentally to produce hyperthermia in laboratory animals [1]. A recent work [2] indicates the possible use of fenfluramine in the treatment of autism. Research is being carried out to determine the effects of fenfluramine on improvement of intelligence of autistic children [3]. It is thought that the action of fenfluramine is through reduction of brain serotonin levels [4]; and fenfluramine has been used to study the effects of serotonin depletion on release of prolactin and growth hormone [5] and on stimulation of adrenocorticotropin secretion in man [6].



I R- C_2H_5

II R- H

III R- C_3H_7

Fig. 1. Structures of compounds cited in the text. I, Fenfluramine; II, norfenfluramine; III, internal standard.

With increases in the clinical applications of fenfluramine, it is necessary to have a quick and reliable method to determine fenfluramine plasma levels. Previous gas—liquid chromatographic (GLC) methods have involved the use of an electron-capture detector and a time-consuming derivatization of the amino group to improve sensitivity and chromatography. Heptafluorobutyric

anhydride [7] and pentafluorobenzoyl chloride [8] derivatization processes require at least a 30-min reaction time and additional sample clean-up steps. Another gas chromatographic method reports a rapid extractive benzoylation of fenfluramine with electron-capture detection, but that method requires a larger sample volume (2 ml) and a higher limit of detection (20 ng/ml) than the method reported here [9]. A recent method assays fenfluramine and norfenfluramine by GLC without derivatization. This method also uses 2-ml sample volumes, requires time-consuming solvent clean-up procedures, and reports lower recoveries for both compounds than our method [10].

Our method uses ethyl chloroformate to derivatize the compounds during the extraction procedure. The elimination of extra derivatization and sample clean-up steps, the use of smaller plasma volumes and the use of the more stable but equally sensitive nitrogen—phosphorus detector are improvements over previous methods.

EXPERIMENTAL

Reagents and materials

Cyclohexane, dichloromethane and methanol were all nanograde solvents (Mallinckrodt, Paris, KY, U.S.A.); ethyl chloroformate (Pfaltz and Bauer, Stamford, CT, U.S.A.) was redistilled before use. Sodium hydroxide, sulfuric acid and ammonium hydroxide (Baker-analysed reagent; J.T. Baker, Phillipsburg, NJ, U.S.A.) solutions were prepared in deionized water.

The internal standard (III, Fig. 1) [N-propyl-α-methyl-3-(trifluoromethyl)-phenethylamine] used was synthesised in our laboratory (A.H. Robins, Richmond, VA, U.S.A.).

Aqueous solutions of fenfluramine and norfenfluramine were diluted in drug-free human plasma to concentrations of 2—100 ng/ml. Aliquots of these plasma standards and all samples were frozen at −20°C until needed for analysis.

Instrumentation

Analyses were performed on a Hewlett-Packard gas chromatograph, Model 5730, equipped with a nitrogen—phosphorus detector. Glass columns, 1.8 m × 4 mm I.D., packed with 3% OV-1 on GCQ, 100—120 mesh (Applied Science Labs., State College, PA, U.S.A.) were conditioned at 200°C overnight with a helium gas flow-rate of 20 ml/min. Chromatographic conditions for the analyses were: column oven temperature, 155°C; inlet temperature, 250°C; helium carrier gas flow-rate, 40 ml/min. Detector temperature was 250°C. Gas flow-rates to the detector were 3.0 ml/min for hydrogen and 50 ml/min for air. Voltage applied to the collector was approximately 19 V.

Assay procedure

Into a 15-ml centrifuge tube, with Teflon® stopper, 0.5 ml of aqueous internal standard solution (200 ng/ml) was placed. A 1-ml aliquot of plasma (standards or unknown samples), 0.5 ml absolute ethanol and 0.5 ml of 5% ammonium hydroxide were added to the tube, then 8 ml of cyclohexane—dichloromethane (3:2) were added and the tube was shaken vigorously for 15 min. The

tube was centrifuged for 10 min at 500 g and then the organic layer was transferred to a clean tube containing 1.0 ml of 0.05 M sulfuric acid. After the sample was shaken for 10 min, the tube was centrifuged. The organic layer was removed and discarded. An additional 5 ml of the extraction solvent was added and the tube vortexed for 10 sec. The organic layer was again removed and discarded. To the acidic layer, 0.5 ml of 2 M sodium hydroxide and 0.3 ml of 5% ethyl chloroformate in cyclohexane—dichloromethane (3:2) were added and the tube was vortexed for 15 sec. A 5-μl aliquot of the reaction mixture was injected into the gas chromatograph.

Chromatographic data were collected and processed electronically (Computer Inquiry Systems, Waldwick, NJ, U.S.A). Peak height ratios of standards were used to calculate unknown concentrations in the samples. There was a 3-min delay in the peak integrations of each chromatogram in order to minimize solvent front effects on data processing.

RESULTS AND DISCUSSION

The extraction efficiency of this method was determined by comparison of peak height ratios of extracted compound to peak height ratios of unextracted standards. It was found the extraction efficiency for fenfluramine (I) was 88% and for norfenfluramine (II) was 91%.

Typical chromatograms of plasma spiked with fenfluramine and norfenfluramine are shown in Fig. 2. There were no interfering chromatographic peaks at the retention time of norfenfluramine (4.1 min), fenfluramine (5.2 min), or the internal standard (6.7 min).

The method was validated to a lower quantitative limit of 2 ng/ml for fenfluramine and 5 ng/ml for norfenfluramine. This validation involved the assay of thirty samples spiked with fenfluramine and norfenfluramine within the concentration range of 0—100 ng/ml (Tables I and II). The concentration of the samples were unknown to the analyst at the time of the assay. All samples were assayed in duplicate.

Six calibration curves were assayed for each compound over a two-week period. These standard curves were linear over the concentration range of 2—100 ng for fenfluramine (r = 0.9997) and 5—100 ng for norfenfluramine (r = 0.9996). The coefficients of variation (C.V.) for points of the standard curve were 3.9% at 2 ng/ml and 1.9% at 100 ng/ml for fenfluramine; and 7.0% at 5 ng/ml and 4.3% at 100 ng/ml for norfenfluramine. The average C.V. for the five points of the standard curve was 3.4% for fenfluramine and 6.8% for norfenfluramine.

Plasma samples from patients dosed with fenfluramine (1.5 mg/kg, per os) were assayed. Fig. 3 shows typical chromatograms from the assay of plasma samples drawn (A) before dosing, (B) 90 min and (C) 180 min after dosing. The concentrations in these samples are 42.5 ng/ml fenfluramine and 6.7 ng/ml norfenfluramine after 90 min and 83.0 ng/ml fenfluramine and 19.2 ng/ml norfenfluramine after 180 min. The pre-treatment sample showed no interfering peaks.

Using chromatographic conditions listed, the structurally related compound d-amphetamine does not have baseline separation from norfenfluramine.

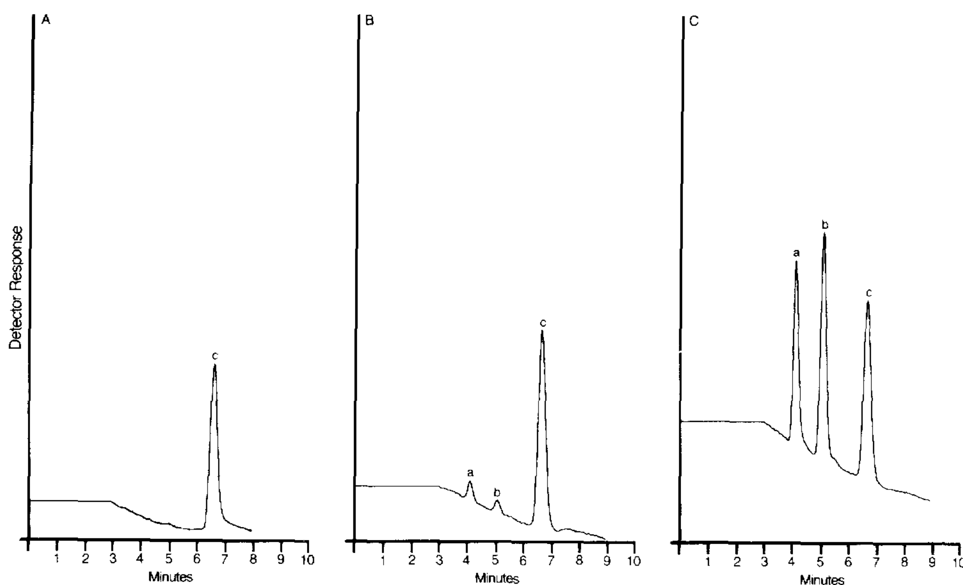


Fig. 2. Chromatograms of 1.0 ml of control plasma spiked with (A) internal stnadard (100 ng); (B) norfenfluramine (5 ng), fenfluramine (2 ng) and internal standard (100 ng); (C) norfenfluramine (50 ng), fenfluramine (50 ng) and internal standard (100 ng). Peaks: a = norfenfluramine; b = fenfluramine; c = internal standard.

TABLE I

DETERMINATION OF ADDED CONCENTRATION OF FENFLURAMINE TO PLASMA

Fenfluramine added as base (ng/ml)	*n*	Mean	S.D.	C.V. (%)	Percentage found
0	6	0.9	0.12	—	—
2	4	2.2	0.21	9.7	110
5	4	5.3	0.12	2.3	106
10	4	10.5	0.22	2.1	105
50	4	53.7	1.31	2.4	107
75	4	79.9	1.98	2.5	107
100	4	106	4.96	4.7	106

TABLE II

DETERMINATION OF ADDED CONCENTRATION OF NORFENFLURAMINE TO PLASMA

Norfenfluramine added as base (ng/ml)	*n*	Mean	S.D.	C.V. (%)	Percentage found
0	6	0.3	0.46	—	—
5	4	5.6	0.49	8.8	113
10	4	11.1	0.18	1.6	111
25	4	25.5	1.47	5.7	102
50	4	56.8	2.05	3.6	114
75	4	81.5	1.46	1.8	109
100	4	105	2.75	2.6	105

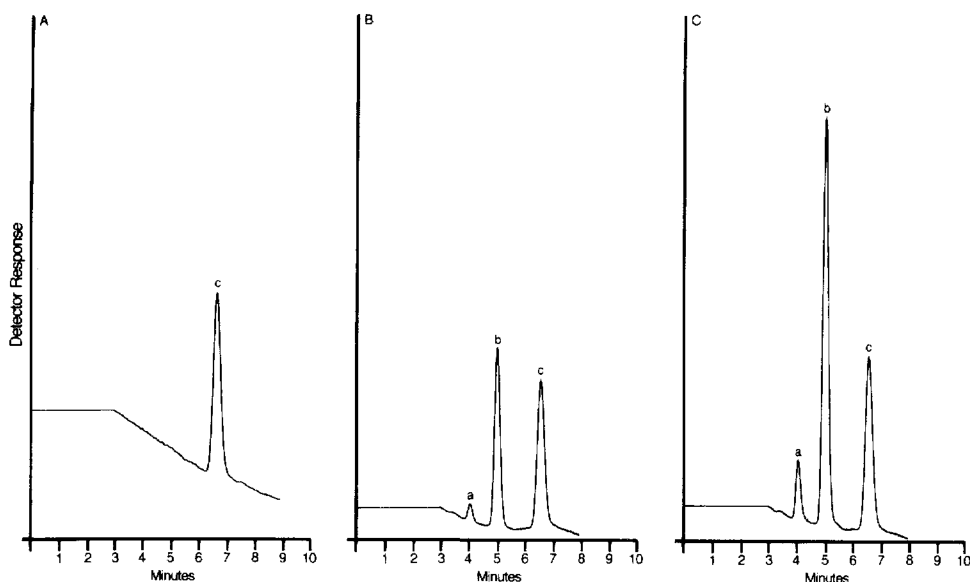


Fig. 3. Chromatograms of patient samples 0 (A), 90 (B) and 180 min (C) after administration of fenfluramine. Peaks: a = norfenfluramine; b = fenfluramine; c = internal standard. See text for explanation.

Relative retention times to fenfluramine are 0.79 for norfenfluramine and 0.84 for *d*-amphetamine. Although not tested, it is thought that methylamphetamine would separate from norfenfluramine and could also be used as the internal standard for this assay.

Ethyl chloroformate reacts with the amine group of the compound to form an amide derivative. This reaction occurs under alkaline conditions during the back-extraction step. The final product has been confirmed by mass spectrometry. This assay procedure and derivatization give chromatograms which are free of any interferences from endogenous plasma constituents.

The demonstrated reliability of this method, the rapid assay procedure and the use of a selective, sensitive detector, make this method applicable to the determination of fenfluramine and norfenfluramine in clinical studies.

ACKNOWLEDGEMENTS

The authors thank David Lewis, M.D., University of Iowa Hospital, Iowa City, IA, U.S.A. for patient plasma samples which were assayed, and Robert E. Lemieux, A.H. Robins Co., Richmond, VA, U.S.A. for synthesis of the internal standard.

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