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New chiral high-performance liquid chromatographic methodology used for the pharmacokinetic evaluation of dexfenfluramine

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Abstract

A new chiral high-performance liquid chromatographic (HPLC) method utilizing ultraviolet (UV) detection has been developed for determining plasma and urinary concentrations of *d*-fenfluramine and its major metabolite *d*-norfenfluramine, while being able to determine the possible presence of *l*-fenfluramine after oral administration of enantiopure *d*-fenfluramine hydrochloride. Sensitivity, stability, and specificity were enhanced by derivatizing the extracted analytes with 3,5-dinitrophenylisocyanate while utilizing a Pirkle-type chiral recognition approach. *In vitro* and *in vivo* statistical data are analogous. Overall plasma inter-assay precision was less than 7% with a minimum quantitation limit of 10 ng/ml. Overall urine inter-assay precision was also less than 7% with a minimum quantitation limit of 25 ng/ml.

1. Introduction

Since the first report of chromatographic separation of optical isomers [1], a large number of methods have been introduced [2–4]. The methods can be divided into three groups [5]: direct separation on chiral columns, separation on achiral columns with chiral mobile phases, and separation of diastereomers formed by pre-column derivatization with chiral reagents. Development of chiral columns has recently been reviewed [6–8], and a number of columns are commercially available [9]. Large separation factors (α) are obtainable in certain cases, but many columns have only moderate efficiency and a number of compounds require derivatization prior to separation, *e.g.* primary amines [10]. Separation methods based on indirect separation of enantiomers by pre-column derivatization with chiral reagents have been reviewed recently [8,11]. The most commonly used reagents for amine compounds are based on isothiocyanate and give ultraviolet (UV) sensitive thiourea derivatives of both primary and secondary amines [12].

Fenfluramine hydrochloride [N-ethyl-,α-methyl-3(trifluoromethyl)benzeneethanamine hydrochloride] is a racemic secondary amine. There is considerable interest in the pharmacology of both the racemate, of the individual enantiomers [*d*-fenfluramine, (I), and *l*-fenfluramine, (II)], and of the corresponding major

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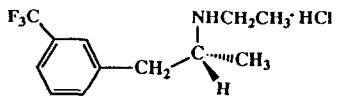
metabolite [*d*-norfenfluramine, (III)] (Fig. 1). Because therapeutic concentrations of I in human plasma are in the low ng/ml range [13] and because the intact drug molecule has low to moderate ultraviolet absorptivity, improving the sensitivity, and specificity, of detection has been a primary analytical challenge. It appears that the literature has but a few references for the analysis of fenfluramine since the initial publication by Campbell [14] in 1970, where he used gas chromatography coupled with flame ionization detection (GC-FID) for the analysis of racemic fenfluramine. Among the most sensitive methods, Caccia and Jori [15] improved upon Campbell's [14] method and reported a gas chromatographic electron capture detection (GC-ECD) method using N-trifluoroacetyl-l-prolyl chloride (TPC) to generate the derivative, or for better ECD response, substituting the pentafluoropropionyl (PPC) group for the trifluoroacetyl group attached to the l-proline yields fenfluramine and norfenfluramine diastereomers which are highly sensitive. These authors reported a linear range of 30 to 300 ng per sample with a minimum detectable amount per injection of 10 pg using a final 1-μl injection volume. This correlates to a minimum detection limit of 10 ng/ml, with a linear dynamic range of 30–300 ng/ml plasma.

(I) d-Fenfluramine HCl



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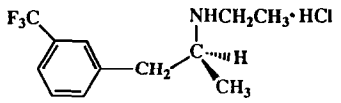
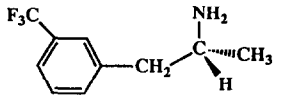


(III) d-Norfenfluramine HCl



(IV) beta-Methylphenethylamine

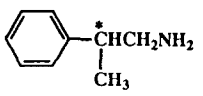


Fig. 1. Basic chemical structures.

Richards *et al.* [16] used a similar approach as Caccia and Jori [15], yet their work involved measurement of I and III in plasma and urine with emphasis on clinical applications. Both methodologies used gas chromatography, while Richards *et al.* used nitrogen specific detection (NSD) instead of ECD. They obtained a lower minimum quantitation limit (2.5 ng/ml), while using a shorter chromatographic run time (4 min), all without the need to derivatize. Plasma calibration curve concentrations (and %C.V.) were from 2.5 (12.0%) ng/ml–100 (3.3%) ng/ml. These advantages would make this method more amenable for the high throughput routine clinical laboratory environment. However, in their paper, it is clearly noted that this method is not stereospecific and is only applicable if separation of the isomers is not required.

In order to have a better understanding of what urinary drug concentrations are to be expected after oral administration of I, Campbell [17] stated that there is little data available on the single enantiomer, yet sufficient data on the racemate. Urinary concentration would be highly dependent on urinary pH (high excretion with low pH), and could vary from as low as 25 ng/ml to as high as 10 μg/ml, from a single 15 mg dose P.O.

However, due to the relatively harsh conditions that gas chromatography requires to volatilize an analyte, and due to the unstable nature of TPC or PPC, reagent and derivative, a less harsh and more stable HPLC method would be preferable.

We tried to develop a sensitive, stereo-specific,

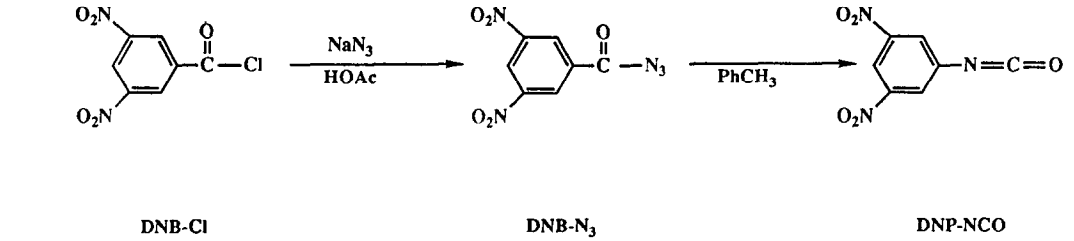
stable HPLC method for the determination of I and III, in addition to II, in human plasma and urine. Several opportunities were considered, and are briefly summarized:

According to Einarsson and Josefsson [18] the reagent (9-fluorenyl)ethylchloroformate (FLEC) can be synthesized from 1-(9-fluorenyl)ethanol (commercially available), normal-phase or reversed-phase HPLC can be applied, sensitivity is supposed to be good (low ng/ml), and the reagent should be stable, yet is very costly.

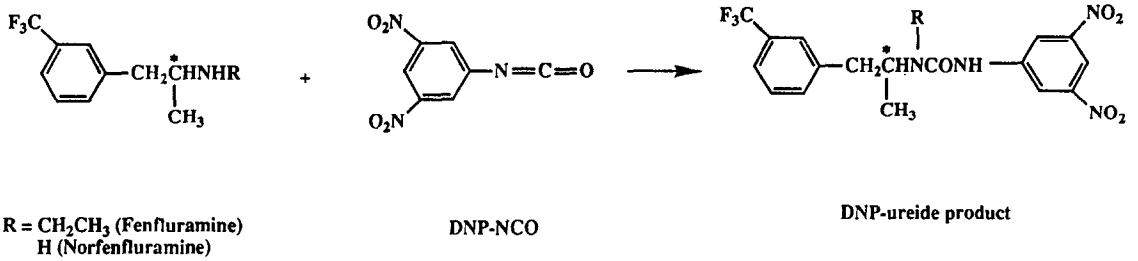
According to Chou *et al.* [19] (9-fluorenyl) methoxycarbonyl-l-proline (FMOC-l-proline) is easier to use than FLEC, since the reagent is commercially available, yet, the reaction kinetics are unsure.

According to Krull *et al.* [20] the reaction kinetics using 3,5-dinitrobenzoyl chloride (DNB-Cl) are fast, but the reagent stability and final sensitivity is questionable (high ng/ml–low μg/ml range). Present work is being directed towards modifying the polymer solid support with efforts of increasing the reagent stability and final sensitivity.

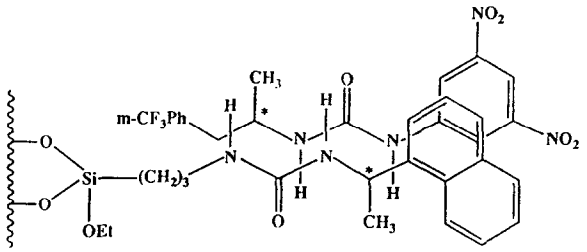
According to Doyle *et al.* [21] 3,5-dinitrophenylisocyanate (DNP-NCO) can be easily synthesized from DNB-Cl (Fig. 2). Sensitivity is good (low ng/ml), the reagent and the ureide



(a)



(b)



(c)

Fig. 2. Synthesis (a), derivatization (b), and proposed chiral recognition mechanism (c).

derivatives are very stable, and a common Pirkle-type column can be employed.

However, all diastereomer approaches have an intrinsic drawback related to uncertain purity and stability of the chiral reagent. Methods involving derivatization with achiral reagents, followed by resolution on HPLC chiral stationary phases (CSP), have also been described [22,23], but have afforded inadequate sensitivity or resolution.

We report here a new, highly sensitive procedure for the resolution of *d*-fenfluramine, the metabolite *d*-norfenfluramine, and the possible impurity *l*-fenfluramine, as the 3,5-dinitrophenyl urea derivatives on a CSP consisting of (*R*)-1-(1-naphthyl)ethyl urea covalently bound to silica through an aminopropyl linkage (Fig. 2). The procedure is based on a CSP originally described by Oi *et al.* [24], which can be prepared by a single *in situ* technique or a commercially available column can be obtained, utilizing a convenient method for urea formation introduced by Pirkle *et al.* [25].

Suitability of this new procedure for the stereo-specific determination of I, II, and III in human plasma and urine was demonstrated by analysis of spiked plasma and urine samples, and has been used for analysis of clinical trial specimens. All reported values are based on using 1.00 ml of heparinized human plasma and 2.00 ml of unpreserved human urine.

For plasma, simultaneous extraction and derivatization were achieved in a one-step process using the DNP-NCO reagent in a reasonable amount of time (1.5 h) at room temperature. The minimum quantitation limit (MQL) was 10 ng/ml plasma, yet the limit of detection (LOD) was 1 ng/ml plasma, per enantiomer. For urine, extraction and derivatization was a two-step process using a mixture of hexane–ethyl acetate, followed by the addition of the reagent. The minimum quantitation limit (MQL) was 25 ng/ml urine, yet the limit of detection (LOD) was 3 ng/ml urine, per enantiomer.

Once extracted, I, II, and III react quantitatively at room temperature with DNP-NCO to form the corresponding 3,5-dinitrophenylureide, having a maximum UV absorption at 235 nm. The formation of ureide derivative serves a number of purposes, all essential to the success of the analytical procedure.

(1) Introduction of the 3,5-dinitrophenyl moiety provides a strong π-acidic group, for complimentary interaction with the π-basic naphthyl group of the CSP. This is essential for effective chiral discrimination.

(2) Formation of the ureide functionally attenuates the polarity of the amino group, improving HPLC efficiency, while retaining hydrogen-bonding potential and introducing a relatively rigid, planar dipole. This also assists in the chiral recognition process.

(3) Additionally, conversion of fenfluramine and norfenfluramine to a non-ionizable, relatively hydrophobic derivative markedly enhances the extraction behavior of the analyte, permitting both extraction and derivatization to be performed in a single step for plasma.

(4) Finally, the high UV absorptivity of the dinitrophenyl group enables detection in the low ng/ml range.

Fig. 2 shows a hypothetical graphical presentation of how the derivative and CSP may come in contact with one another in order for effective chiral discrimination to occur.

Isomeric elution order was established by derivatization and chromatography of a sample of enantiomerically pure (optical purity of >99%) I and III, while comparing the retention characteristics of racemic fenfluramine. It is also known that chiral amines are generally resistant to racemization under such conditions [26].

This HPLC procedure achieves the required resolution of the enantiomeric ureide derivatives of I, II, and the major metabolite III, without the need for diastereomer derivatization. The CSP is easily prepared, or is obtainable through commercial sources. The derivatization procedure is experimentally convenient, achieving both extraction and derivatization within a reasonable amount of time and at room temperature. Sensitivity, via UV detection, is adequate for expected levels, without the need for concentrating the sample.

Results on spiked human plasma, urine samples and real clinical trial specimens demonstrate

the absence of interferences and exhibit the expected analytical response.

2. Experimental

2.1. Reagents

Acetonitrile, hexane, isopropanol, methylene chloride, and toluene (all HPLC grade) were from Baxter (Edison, NJ, USA). Acetic acid (HPLC grade), hydrochloric acid, (reagent grade), and ethyl acetate (HPLC grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA). The compounds I, *d,l*-fenfluramine hydrochloride, and III (all reference standards) were from Servier Technologie (Paris, France). 3,5-Dinitrobenzoylchloride (>98%), sodium azide (>99.5%) and trichloroacetic acid (>99.5%) were purchased from Fluka (Ronkonkoma, NY, USA). Human plasma, drug-, HIV-, and HBV-free control were purchased from Biological Specialities, (Lansdale, PA, USA). Human urine [drug-free control (50% male/50% female, v/v) was in-house pooled (Roche Biomedical Laboratories, Raritan, NJ, USA). Sodium hydroxide (analytical reagent grade) was purchased from Mallinckrodt (Paris, KY, USA). Water (HPLC grade) was produced in-house, Milli-Q/HPLC grade.

2.2. Equipment

Ordinary laboratory equipment was used throughout the sample preparation and derivatization process.

The HPLC systems were assembled using modular components from various manufacturers, and were the CM4100 analytical solvent delivery systems, SM3200 variable wavelength UV detectors, Chromjet integrators – for archival purposes only (Thermo Separation Products, Riviera Beach, FL, USA; formerly known as LDC Analytical), 715 UltraWISP autosamplers (Waters, Milford, MA, USA), column heater (Alltech, Deerfield, IL, USA) and saturator (placed post-pump/pre-injector). Analytical columns were Silica, 250 × 4.6 mm I.D., 12 μm particle size, and (*R*)-naphthylurea Chiral, 250 × 4.6 mm I.D., 5 μm particle size, respectively (Supelco, Bellefonte, PA, USA).

2.3. Computerization

The computerized data acquisition and processing system contained the PENelson 2600 software package with 970 series interfaces (PENelson, Paramus, NJ, USA), IBM Model 95 PCs (IBM, Armonk, NY, USA), DMLIMS + software package (PennComp, Ardmore, PA, USA), RS/1 software package (BBN, Cambridge, MA, USA), and WordPerfect 5.0 software package (WordPerfect, Orem, UT, USA).

All calculations were based on peak-height ratios; the peak height of the standard was divided by the peak height of the internal standard (1st enantiomer peak).

Concentrations of I, II, and III in the quality assurance and clinical trial samples were determined using the slope (A) and intercept (B) values of a line estimated by weighted least squares regression (weighted as $1/y$) of the peak ratio *versus* concentration from the calibration standards. All concentrations were reported as if the analytes were in the salt (HCl) form.

DMLIMS+ (Drug Metabolism Laboratory Information Management System) was used as a data coordination system. Its function was to assist the analyst(s) in all aspects of drug metabolism studies from protocol design to data analysis to the reporting of data. During the conduct of a study or protocol, DMLIMS+ creates and maintains an electronic record of all important steps and automates many manual steps in a drug metabolism study. The program automates the design, sample handling, sample scheduling, sample analysis, and reporting of bioanalytical and pharmacokinetic analyses and will serve as the basis for automation of record keeping.

2.4. Preparation of 3,5-dinitrobenzoyl azide (DNB-N_3)

See Fig. 2 for schematic representation. Combine 100 ml (1.75 moles) of glacial acetic acid and 6.40 g (0.028 mole) of 3,5-dinitrobenzoyl

chloride (DNB-Cl) in a flask equipped with a magnetic stir bar. Sodium azide (1.80 g, 0.028 mole) was added in small increments. Continue stirring for 1 h. The solution should gradually become turbid. The product (DNB-N_3) was precipitated by the addition, with continuous stirring, of 300 ml of cold HPLC-grade water. The precipitate was suction filtered, washed with small portions of HPLC-grade water, and dried overnight. Yield = 62%. The white, crystalline, non-hygroscopic product was stored in a vacuum desiccator filled with indicating Drierite.

2.5. Preparation of 3,5-dinitrophenylisocyanate (DNP-NCO)

See Fig. 2 for schematic representation. Using a 50-ml class A volumetric flask, 25.0 mg of DNB-N_3 was dissolved in 5 ml of toluene and refluxed on a hot plate for 10 min. The flask was cooled to room temperature and then filled to the mark with methylene chloride.

A portion of this DNP-NCO solution was diluted with methylene chloride, 1:200 (v/v) for plasma, and 1:100 (v/v) for urine, to give a final working solution concentration of approximately $1 \cdot 10^{-5}$ *M*, and $2 \cdot 10^{-5}$ *M*, respectively. The DNP-NCO solutions were prepared fresh daily.

2.6. Standard solution preparation

All sample preparations and analyses were conducted under yellow lighting in order to avoid possible sample degradation.

Analytical and internal standard stock solutions of I, II, III, and beta-methylphenethylamine (IV, internal standard see Fig. 1) were prepared in HPLC-grade water. Concentrations for plasma were 40 μg/ml for I, II, and III, and 1.25 μg/ml for IV. The same concentrations were used for urine, except IV was 5.0 μg/ml.

Working solutions were prepared in HPLC-grade water by appropriate serial dilution of stock solutions. Final concentrations for plasma were 4.00, 2.00, 1.00 μg/ml, and 500, 250 and 100 ng/ml for I, II, and III. Final concentrations for urine were 10.00, 5.00, 2.00 and 1.00 μg/ml, and 500 and 250 ng/ml for I, II, and III.

2.7. Quality assurance sample preparation

Quality assurance (QA) samples were used to assure the method was reliable and consistent from project onset through completion. These samples were designed to emulate actual clinical trial specimens, whereby their known *versus* determined concentrations should not vary by more than a predetermined percentage throughout the entire study.

QAs were prepared separately from the above standard stock and working solutions. A QA stock solution of 40 μg/ml in water was prepared. This was diluted to an intermediate concentration of 1 μg/ml in water.

For plasma, QA low (20 ng/ml) and QA high (80 ng/ml) were prepared by aliquoting small volumes from the intermediate concentration directly into drug-free control human plasma. Aliquots (1.00 ml) of QA low and QA high were stored frozen at −70°C until assayed. Two QA low and two QA high were assayed with each autosampler tray.

For urine, QA low (70 ng/ml) and QA high (300 ng/ml) were prepared by aliquoting small volumes of the intermediate concentration solution directly into drug-free control human urine. Aliquots (2 ml) of QA low and QA high were stored frozen at −70°C until assayed. Again, two QA low and two QA high were assayed with each tray.

2.8. Standard curve

For plasma, each calibration curve was generated with 1.00 ml of drug-free control human plasma fortified with 10.0, 25.0, 50.0, 100, 200, and 400 ng/ml of I, II, and III. Internal standard concentration (IV) was maintained at 125 ng/ml.

For urine, each calibration curve was generated with 2.00 ml of drug-free control pooled-human urine fortified with 25.0, 50.0, 100, 200, 500, and 1000 ng/ml of I, II, and III. Internal standard concentration (IV) was maintained at 500 ng/ml.

2.9. Sample preparation

Plasma

Plasma control blanks were prepared by aliquoting 1.00 ml of drug-free control human plasma into a 10-ml disposable centrifuge tube and adding 300 μl of HPLC-grade water.

To 1.00 ml of previously aliquoted clinical trial and QA specimens, add 100 μl of internal standard solution (1.25 μg/ml of IV) and 200 μl HPLC-grade water.

All samples were vortex-mixed, 400 μl of 1 *M* trichloroacetic acid was added, and again vortex-mixed. The samples were then centrifuged at 3000 rpm (2500 *g*) for 10 min. A 900-μl aliquot of the clear, aqueous top layer was then quantitatively transferred to a clean 10-ml centrifuge tube. NaOH (250 μl, 1 *M*) was added to each tube. Derivatizing reagent (2 ml of DNP-NCO working solution) was added to each tube, vortex-mixed) for 2 s, and the reaction was allowed to go to completion (1.5 h). The aqueous (top) layer was carefully discarded. A 1.00 ml volume of the organic (bottom) layer was quantitatively transferred to a clean 10-ml disposable centrifuge tube where it was evaporate to dryness under a stream of dry nitrogen. The sample was reconstituted with 200 μl of methylene chloride and loaded into 1-ml HPLC autosampler vials containing 100 μl inserts. The vials were securely capped to prevent further evaporation.

Urine

Urine control blanks were prepared by aliquoting 2.00 ml of drug-free control pooled human urine into a 10-ml disposable centrifuge tube and adding 600 μl of HPLC-grade water.

To 2.00 ml of previously aliquoted clinical trial and QA specimens, add 200 μl of internal standard solution (5.00 μg/ml of IV) and 400 μl HPLC-grade water.

All samples were vortex-mixed for 5 s, 400 μl of 1 *M* NaOH was added, and again vortex-mixed. The samples were then centrifuged at 2500 *g* for 10 min. The clear, aqueous top layer was decanted into a clean 15-ml centrifuge tube. Extraction solvent mixture (5 ml, hexane–ethyl acetate, 70/30, v/v) was added to each tube and the tubes were shaken gently by hand for 1 min and allowed to stand for 10 min. A 4.00-ml volume of the upper organic layer was quantitatively transferred to a clean 10-ml disposable centrifuge tube and evaporated by a stream of dry nitrogen to a final volume of *ca.* 30 μl (analytes and internal standard are volatile prior to derivatization). Derivatizing reagent (1 ml of DNP-NCO working solution) was added to each tube, vortex-mixed for 2 s, and the reaction was allowed to go to completion (0.5 h). The final extract was equally split into separate 1-ml HPLC autosampler vials.

2.10. HPLC conditions

Individual solvent components were degassed under conditions of high vacuum and bumping, and then filtered through a 0.2-μm nylon (or equivalent) membrane. The following ratios of hexane to isopropanol may need to be adjusted slightly (±2–3%, in 0.5% increments) in order to achieve adequate resolution amongst the analytes of interest due to minor HPLC column batch-to-batch differences.

Plasma

Mobile phase was prepared accurately and precisely as a mixture of HPLC grade hexane–isopropanol–acetonitrile (89.0:9.0:2.0, v/v/v). The resultant solution was further mildly mixed via magnetic stirring.

Chromatographic run time and flow programming was: 0–25 min at 1.20 ml/min, 25.1–38 min at 3.50 ml/min (wash period), and 38.1–45 min at 1.20 ml/min (equilibration period). Detection was at 235 nm at a sensitivity of 0.002 AUFS. A 35-μl aliquot of the final extract was injected. Retention times for I, II, III, and IV (first peak) were approximately 10.0, 11.5, 15.6, and 20.7 min, respectively (Figs. 3 and 4).

Urine HPLC system A (I and III)

Mobile phase was prepared accurately and precisely as a mixture of HPLC grade hexane–isopropanol–acetonitrile (90.5:7.5:2.0, v/v/v). The resultant solution was further mildly mixed via magnetic stirring.

Chromatographic run time and flow-rate was 25 min at 1.20 ml/min. The column temperature was 35°C. Detection was at 235 nm using a sensitivity of 0.002 AUFS. A 35-μl aliquot of the final extract was injected. Retention times for I, III and IV (first peak) were approximately 9.3, 15.1, and 19.5 min, respectively (Figs. 5 and 7).

Urine HPLC system B (II)

Mobile phase was prepared identically as that for plasma.

Chromatographic run time and flow-rate was 25 min at 1.20 ml/min. The column was at ambient temperature. Detection was at 235 nm using a sensitivity of 0.002 AUFS. A 35-μl aliquot of the final extract was injected. Retention times for II and IV (first peak) were approximately 10.7 and 18.8 min, respectively (Figs. 6 and 8).

3. Results and discussion

The chiral *S* column yielded a favorable separation of II from I over the *R* column, when using pure standards dissolved in mobile phase, since II eluted prior to I and the reagent. This would have been preferable if one were analyzing a clean product and interested in determining the % II as compared to I. However, when extracted from plasma, a strongly retaining compound eluted at approximately 80 min. Because of this impractical run time, the *R* column was chosen over the *S* column, where run time was roughly reduced by 50%. The *R* column allowed us to use a stronger (more polar) mobile phase where run time was reduced while retaining adequate resolution among the analytes. Fig. 3 shows an example of a blank chromatogram (bottom, denoted S-0), standard 1 having a 10 ng/ml concentration of I, II, and III (middle, denoted S-1), and standard 3 having a 50 ng/ml concentration of I, II, and III (top, denoted S-3).

Extreme care was required in mobile phase preparation. Differences of ±0.5% had a significant effect on the retention and resolution of I, reagent peak and II. The highly specific, yet delicate, balance between the interactions of the diastereomers with the CSP must be well main-

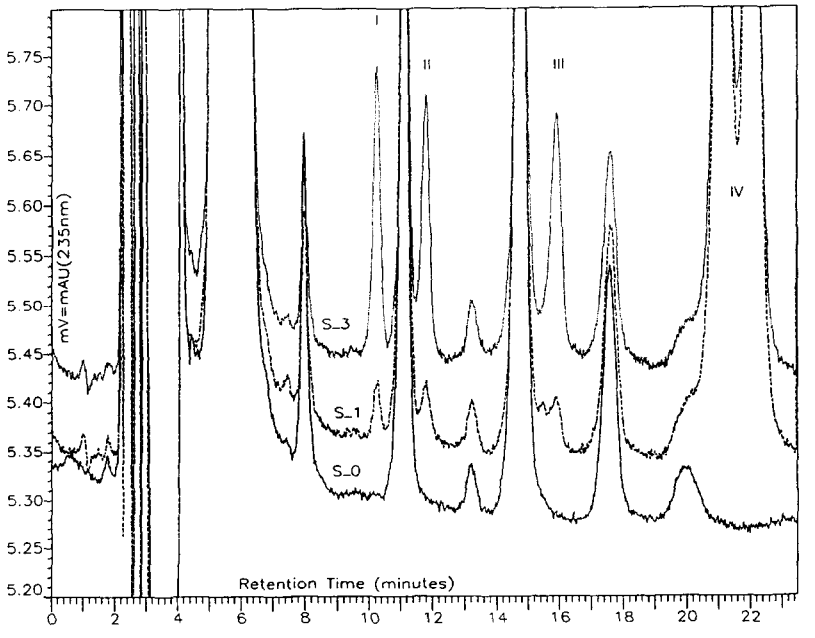


Fig. 3. Chromatographic overlays of drug-free control plasma (S-0); drug-free control plasma fortified with 10 ng/ml of I, II, and III, and 125 ng/ml of internal standard IV (S-1); and drug-free control plasma fortified with 50 ng/ml of I, II, and III, and 125 ng/ml of internal standard IV (S-3).

tained. In addition, residual water had an equal role in retention, resolution, and column longevity. In order to prevent stationary-phase changes, we incorporated a silica "saturator" column placed post-HPLC pump/pre-injector. The saturator column was helpful by removing residual water from the mobile phase prior to contact with the analytical column, and by increasing HPLC-system back pressure, permitting this method to be reliable and sensitive.

Overall sensitivity was adequate for multi-dose and high single-dose pharmacokinetic evaluations. When we began this project several general schemes were available to us, however, the methodology as proposed by Doyle [21], seemed to be the most logical way to initially proceed.

Possible variations from Doyle's methodology were investigated with efforts addressed to increasing assay sensitivity and decreasing the minimum quantitation limit (MQL). The MQL was determined by taking into account an adequate average signal-to-noise (S/N) ratio between 5 and 10, and also taking into account its quantitatability. Quantitatability is further defined by having the ability to consistently yield a value that is within a certain error margin when compared to the theoretical value. When multiple instruments were used in a given protocol, slight differences in the resultant statistical values were inherent, and were closely monitored and evaluated if they were to be considered valid.

Commercially available columns gave us the most consistent chromatographic behavior, yet the *in situ* column gave us comparable results. With efforts addressed at reducing both MQLs, narrow-bore (2 mm) and micro-bore (1 mm) columns were investigated. Though solvent consumption was reduced over that of conventional-bore columns (4.6 mm), the MQL appeared to be unchanged. More in-depth investigation would be needed to verify our initial qualitative findings.

Several combinations of extraction solvents were investigated. For plasma, DNP-NCO dissolved in methylene chloride was superior to all other combinations with respect to the trade-off between analyte recovery and chromatographic interferences. However, for urine, this extraction process was more complicated. A mixture of hexane–ethyl acetate (70:30, v/v) as the extraction solvent, and then derivatizing with DNP-NCO (a two-step process), gave us adequate recovery. Physical determinations for total % recovery were omitted, yet could have been performed via radio-labelling techniques.

Therapeutic plasma concentrations were known to be in the low ng/ml range, therefore, quantitation was validated to cover the range 10–400 ng/ml. However, anticipated urinary concentrations were highly speculative and two linear ranges were developed (10–400 ng/ml and 25–1000 ng/ml). The latter was further investigated and validated. The urine method was more complicated than that for plasma and chromatographic resolution of the analytes of interest *versus* interferences was our major difficulty. Two slightly different chromatographic conditions were required in order to quantitate within acceptable error limits.

In order to determine if experimental conditions, *i.e.* selectivity (α), capacity factor (k'), theoretical plates (N), and subsequently resolution (R_s), were appropriate prior to the onset of any tray, test mixes (usually the high standard) were run in order to determine if the various separation factors were adequate. Once determined to be acceptable, experimental analyses on clinical trial specimens commenced. Typical overall physical characteristics of the separation were in the order of that expected, where α ranged from 0.6–1.2 (analyte peak and closest neighbor), k' from 3.0 to 6.8 (using t_0 equal to the system dead volume in units of minutes), and $N_{1/2h}$ from 6700–9600 plates/column.

The plasma calibration curves and resultant data yielded linear, precise and accurate data. Table 1 shows the overall inter-assay precision for standards and QAs used for the initial validation and Table 2 shows the clinical trial data. The overall C.V. was less than 7% for all. Calibration curve data for the initial validation produced a minimum mean coefficient of determination, R^2 (correlation coefficient squared), of >0.994 with a slope C.V. of $<8\%$, while for the clinical trial study $R^2 > 0.997$ with a slope C.V. of $<13\%$. The higher slope C.V. for the clinical trial study was due to using multiple

Table 1
Summary of inter-assay precision from the analysis of the calibration curve and QA sample data during the plasma validation

	STD-10	STD-25	STD-50	STD-100	STD-200	STD-400	QA-20	QA-80
d-Fenfluramine hydrochloride (I)								
Mean	10.84	23.69	47.44	98.54	210.2	397.2	21.64	76.85
S.D.	0.66	0.59	2.05	7.25	18.2	19.4	1.38	5.54
C.V. (%)	6.09	2.49	4.32	7.36	8.66	4.88	6.38	7.21
n	3	3	3	3	3	3	3	3
Overall C.V. (%)	5.92							
l-Fenfluramine hydrochloride (II)								
Mean	10.87	23.43	48.10	98.27	210.5	397.2	21.91	77.46
S.D.	0.69	0.68	2.78	8.25	18.97	20.64	1.69	5.24
C.V. (%)	6.35	2.90	5.78	8.40	9.01	5.20	7.71	6.76
n	3	3	3	3	3	3	3	3
Overall C.V. (%)	6.51							
d-Norfenfluramine hydrochloride (III)								
Mean	10.49	24.10	48.13	100.5	206.0	397.1	20.21	79.08
S.D.	0.28	0.37	1.88	5.17	11.45	12.47	1.19	4.07
C.V. (%)	2.67	1.54	3.91	5.14	5.56	3.14	5.89	5.15
n	3	3	3	3	3	3	3	3
Overall C.V. (%)	4.13							

All concentration units are in ng/ml. STD = analytical standard concentration. QA = quality assurance concentration.

Table 2
Summary of inter-assay precision from the analysis of the calibration curve and QA sample data during the plasma clinical trial

	STD-10	STD-25	STD-50	STD-100	STD-200	STD-400	QA-20	QA-80
d-Fenfluramine hydrochloride (I)								
Mean	10.00	25.06	50.04	101.0	200.5	399.7	20.52	80.13
S.D.	0.64	1.76	2.66	5.32	10.31	8.87	1.62	5.59
C.V. (%)	6.40	7.02	5.32	5.27	5.14	2.22	7.89	6.98
n	46	45	45	45	45	46	46	46
Overall C.V. (%)	5.78							
l-Fenfluramine hydrochloride (II)								
Mean	10.02	25.09	50.13	100.6	200.7	399.7	20.54	80.50
S.D.	0.86	1.73	2.59	5.57	10.26	8.62	1.52	5.75
C.V. (%)	8.58	6.90	5.17	5.54	5.11	2.16	7.40	7.14
n	44	46	45	45	45	46	46	46
Overall C.V. (%)	6.00							
d-Norfenfluramine hydrochloride (III)								
Mean	10.40	24.89	48.32	100.0	202.4	399.9	20.86	80.54
S.D.	0.58	1.32	2.38	5.09	6.85	7.44	1.41	4.05
C.V. (%)	5.58	5.30	4.93	5.09	3.38	1.86	6.76	5.03
n	46	46	45	45	45	46	46	46
Overall C.V. (%)	4.74							

All concentration units are in ng/ml. STD = analytical standard concentration. QA = quality assurance concentration.

Table 3
Summary of inter-assay precision from the analysis of the calibration curve and QA sample data during the urine validation

	STD-25	STD-50	STD-100	STD-200	STD-500	STD-1000	QA-70	QA-300
d-Fenfluramine hydrochloride (I)								
Mean	25.0	50.2	101.3	199.8	489.0	1011	72.3	309.9
S.D.	1.4	1.6	2.1	2.8	12.8	17.2	7.2	16.5
C.V. (%)	5.60	3.19	2.07	1.40	2.62	1.70	9.96	5.32
n	4	4	4	4	4	4	4	4
Overall C.V. (%)	3.98							
l-Fenfluramine hydrochloride (II)								
Mean	25.2	50.1	101.5	198.5	491.7	1009	69.1	309.7
S.D.	1.8	2.3	1.8	2.8	10.2	12.8	5.2	10.9
C.V. (%)	7.14	4.59	1.77	1.41	2.07	1.27	7.53	3.52
n	4	4	4	4	4	4	4	4
Overall C.V. (%)	3.66							
d-Norfenfluramine hydrochloride (III)								
Mean	25.2	48.3	102.4	210.1	480.9	1011	69.5	303.0
S.D.	1.7	1.7	6.9	9.2	13.0	14.4	6.6	17.7
C.V. (%)	6.75	3.52	6.74	4.38	2.70	1.42	9.50	5.84
n	4	4	4	4	4	4	4	4
Overall C.V. (%)	5.11							

All concentration units are in ng/ml. STD = analytical standard concentration. QA = quality assurance concentration.

instruments and staff over a 4 month period. Analogous data was generated for urine and is shown in Tables 3 and 4.

A question that needs to be addressed in these types of studies, is that of sample stability, both of the final extract (short term bench-top stability) and that of the specimen from the time the specimen is actually collected from the patient to eventual laboratory assaying (short and long term storage stability). The final extract stability tests were conducted (in triplicate) by preparing multiple aliquots of a single concentration plasma QA sample, and comparing the resultant values over time to that of the newly generated standard calibration curves. This data showed that the extract was stable while in the autosampler, at ambient laboratory conditions, for up to a minimum of 72 h (C.V. typically less than 10%), yet 48 h was never exceeded during the protocol. Short and long term plasma storage stability data was conducted by monitoring values generated by the QAs throughout the study, and showed that these specimens were stabile, at −70°C, for a minimum of 4 months.

Fig. 4 shows randomly selected patient plasma chromatograms illustrating typical concentrations that were evidenced during the clinical trial. The bottom chromatogram (denoted P-0) is a control blank, showing the absence of possible chromatographic interferences at concentrations we can quantitate. The second from the bottom (denoted P-1) shows a low concentration chromatogram having a I and III concentration of 20.3 ng/ml and N.M. (non-measurable, having an extrapolated value of 8.9 ng/ml) which is below our minimum quantitation limit, respectively. The second from the top chromatogram (denoted P-2) shows a I and III concentration of 27.7 and 12.1 ng/ml, respectively. One of the highest concentrations determined was that of the top chromatogram (denoted P-3) where I and III were 88.8 and 59.9 ng/ml. II was not evidenced, nor quantitatable, for all patient plasma chromatograms. This does not prove that II was not present in the plasma, yet if it were present, its concentration was lower than this method could determine. Because of this, possible physiological interconversion (I to II) was also not evident.

As mentioned previously, the urine assay

Table 4
Summary of inter-assay precision from the analysis of the calibration curve and QA sample data during the urine clinical trial

	STD-25	STD-50	STD-100	STD-200	STD-500	STD-1000	QA-70	QA-300
d-Fenfluramine hydrochloride (I)								
Mean	22.7	49.9	105.7	209.8	506.1	984.6	66.9	279.6
S.D.	1.8	3.4	6.4	12.6	14.6	12.0	5.1	16.1
C.V. (%)	7.93	6.81	6.05	6.01	2.88	1.22	7.62	5.76
n	21	20	19	20	21	21	21	21
Overall C.V. (%)	5.54							
l-Fenfluramine hydrochloride (II)								
Mean	23.4	50.1	103.3	206.5	506.4	987.8	65.7	278.5
S.D.	2.2	1.8	6.3	11.9	14.0	13.4	4.9	20.1
C.V. (%)	9.40	3.59	6.10	5.76	2.76	1.36	7.46	7.22
n	17	15	15	17	17	17	17	17
Overall C.V. (%)	5.46							
d-Norfenfluramine hydrochloride (III)								
Mean	23.3	49.8	104.1	210.5	501.2	989.9	70.9	291.6
S.D.	2.1	3.9	7.9	12.8	18.5	15.7	5.6	18.9
C.V. (%)	9.01	7.83	7.59	6.08	3.69	1.59	7.90	6.48
n	21	20	20	20	21	21	21	21
Overall C.V. (%)	6.27							

All concentration units are in ng/ml. STD = analytical standard concentration. QA = quality assurance concentration.

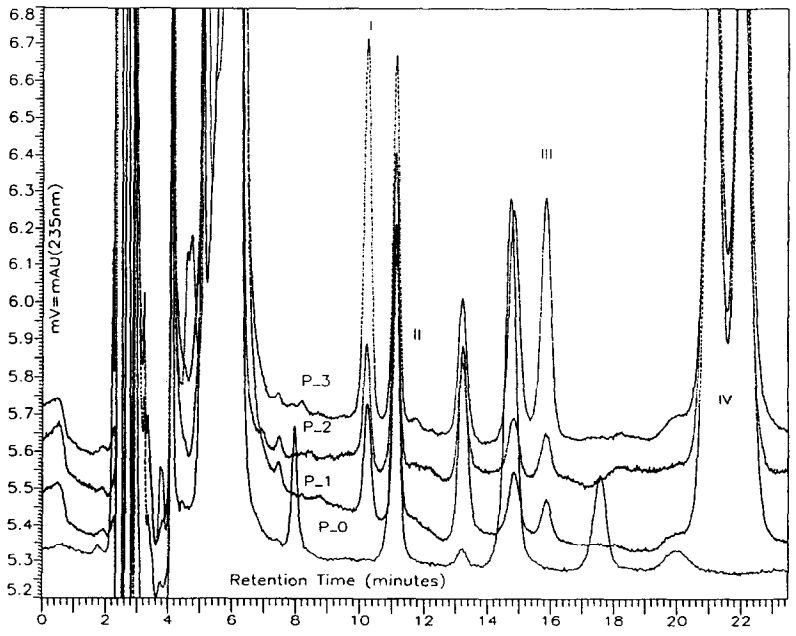


Fig. 4. Clinical trial plasma specimens illustrating the following typical chromatograms; control blank (P-0), low concentration (P-1), intermediate concentration (P-2) and high concentration of drug (P-3).

required splitting the final extract for subsequent analysis on dual HPLC systems, each having slightly different chromatographic conditions (urine HPLC systems A and B). Urine HPLC system A was used for the quantitation of I and III, since II co-eluted with a plasma/reagent peak at approximately 10.2 min. It is interesting to note that even though it is apparent that urine HPLC system B was able to separate I, II, and III, under a single set of chromatographic conditions, adequate baseline resolution was not possible and, therefore, only analyte II could be quantitated within our predetermined error limit. Figs. 5 (HPLC system A) and 6 (HPLC system B) show examples of a blank urine chromatogram (bottom, denoted S-0), standard 1 having a 25 ng/ml concentration of I, II, and III (middle, denoted S-1), and standard 3 having a 100 ng/ml concentration of I, II, and III (top, denoted S-3).

Fig. 7 shows randomly selected patient chromatograms for I and III, while Fig. 8 shows the corresponding patient chromatograms for II. A control blank is illustrated on the bottom of both figures as P-0. Random specimen No. 1 (denoted as P-1) had urinary concentrations for I, II and III of 51.7, N.M. (non-measurable), and 198.8 ng/ml, respectively. Random specimen No. 2 (denoted as P-2) had urinary concentrations for I, II and III of V.E.C. (value exceeds calibration, extrapolated value was 2300 ng/ml which required reassaying via dilution with matrix), 40.5, and 719.6 ng/ml, respectively. Random specimen No. 3 (denoted as P-3) had urinary concentrations for I, II and III of V.E.C. (value exceeds calibration, extrapolated value was 5300 ng/ml which required reassaying via dilution with matrix), 99.6, and 750.2 ng/ml, respectively. In addition, the percent II as compared to I in random specimens No. 2 and No. 3, were approximately 1.8%. This data coincides with what was determined in a later assay developed by our laboratory to quantitate I and II in the final pharmaceutical product [27], hence, the possibility of physiological interconversion, again, was not apparent.

Figs. 9 and 10 show selected pharmacokinetic profiles for a random patient receiving a single 30 mg dose (P.O.) and another random patient receiving multiple 15 mg doses (P.O./B.I.D.)

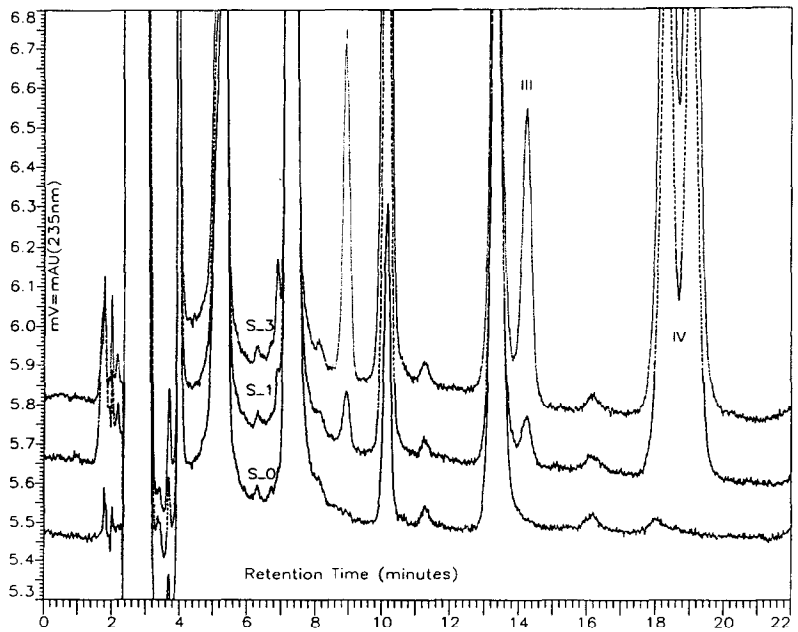


Fig. 5. Chromatographic overlays of drug-free control urine; drug-free control urine spiked with 25 ng/ml of I and III, and 500 ng/ml of IV; and drug-free control urine spiked with 100 ng/ml of I and III, and 500 ng/ml of IV.

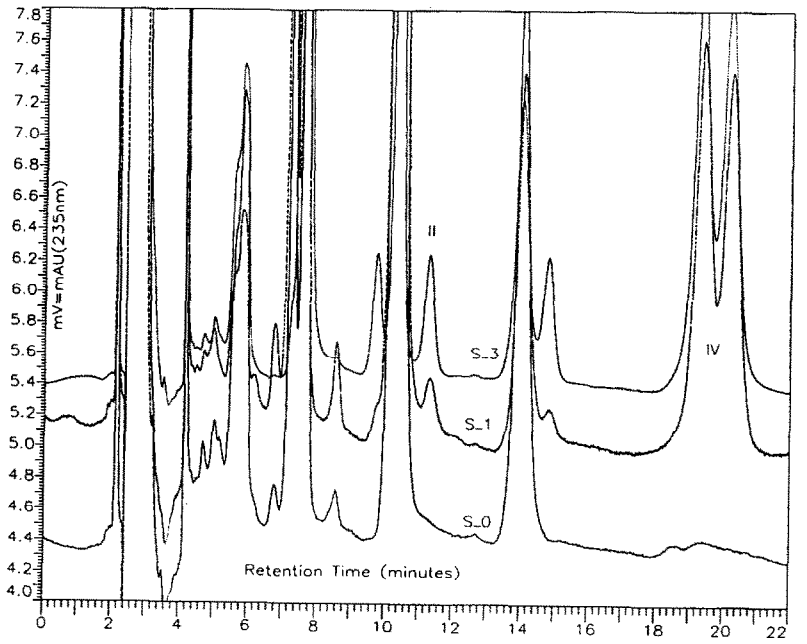


Fig. 6. Chromatographic overlays of drug-free control urine; drug-free control urine spiked with 25 ng/ml of II and 500 ng/ml of IV; and drug-free control urine spiked with 100 ng/ml of II and 500 ng/ml of IV.

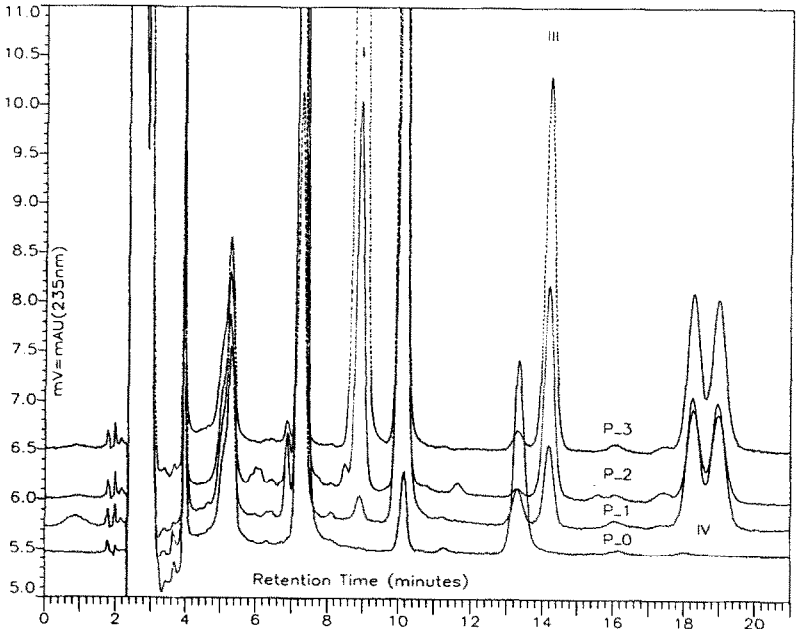


Fig. 7. Chromatographic overlays of random clinical trial chromatograms from HPLC system A (I and III) illustrating the following: control blank (P-0), low concentration (P-1), medium concentration (P-2), and high concentration (P-3).

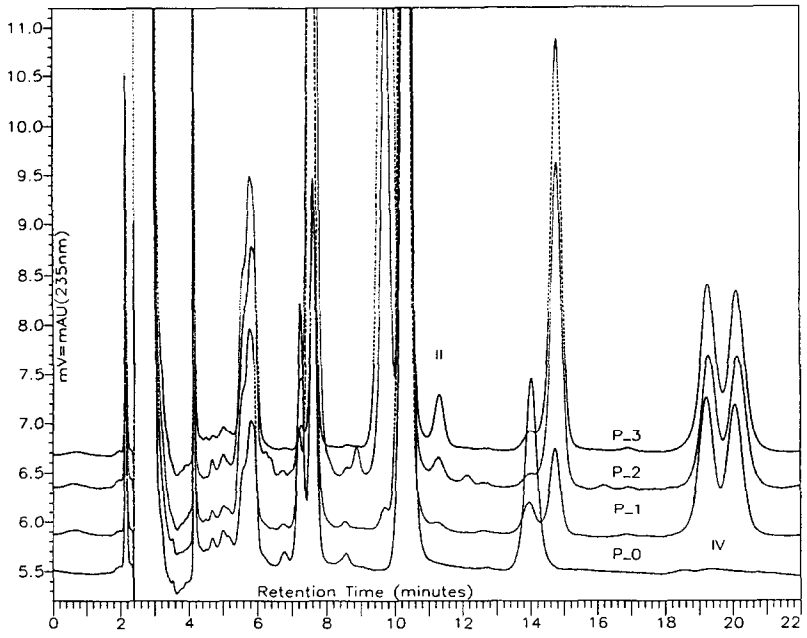


Fig. 8. Chromatographic overlays of random clinical trial chromatograms from HPLC system B (II) illustrating the following: control blank (P-0), low concentration (P-1), medium concentration (P-2), and high concentration (P-3).

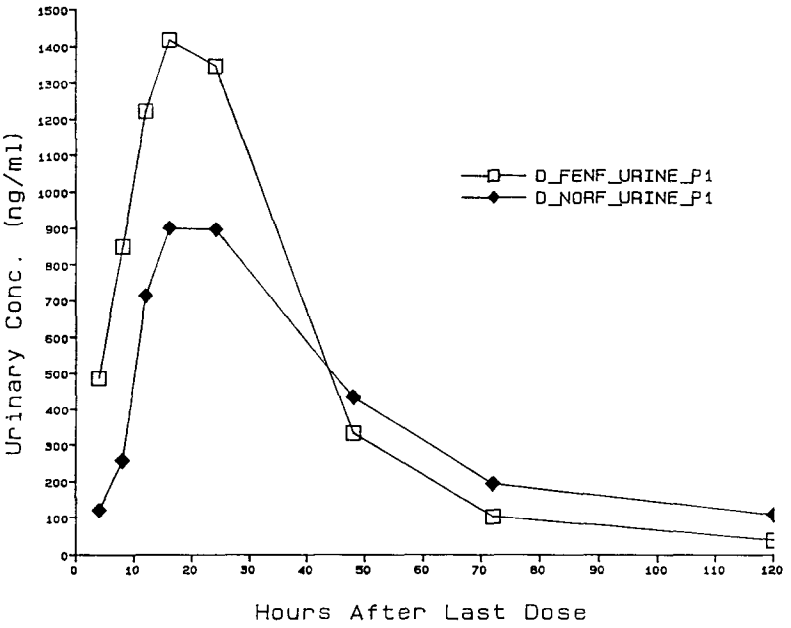


Fig. 9. Random pharmacokinetic profile after a single 30 mg dose (p.o.) of I.

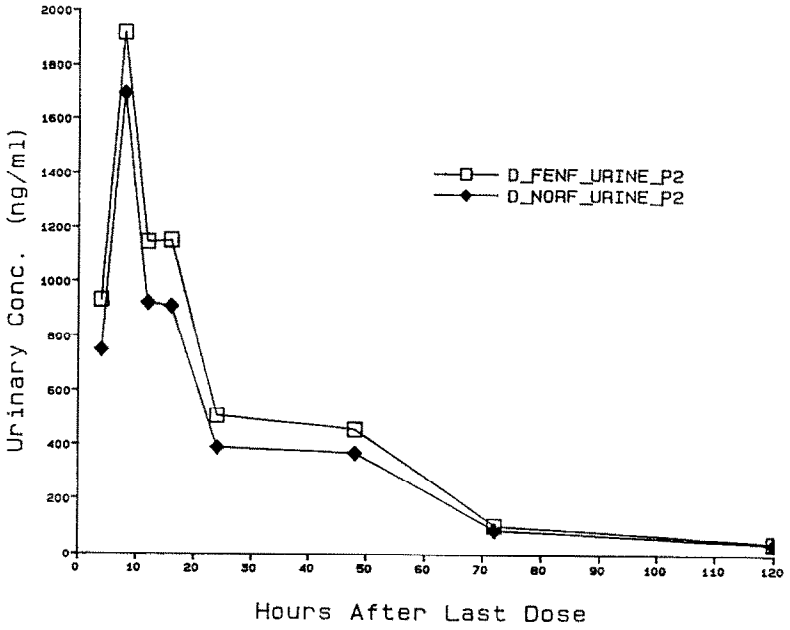


Fig. 10. Random pharmacokinetic profile of multiple 15-mg doses (p.o./b.i.d.) of I, over 2 weeks, while monitoring 120 h after the last dose.

over a two week period, respectively. Specimen collection began after the last oral dose was received, and proceeded for 120 h. Note that mass recovery was not calculated, yet could have been performed, by incorporating total voided urine volume at a given collection time. These plots show that our methodology can be used for monitoring human patients receiving orally administered *d*-fenfluramine hydrochloride.

It is also interesting to note that when additional studies were undertaken [27], some interesting qualitative findings were noticed. Not only was the reagent DNP-NCO applicable to primary and secondary amines, it appears that it may also be applicable to some alcohols. When alternate mobile phases were investigated and isopropanol was replaced with methylene chloride, the large peak that we attributed to the reagent, and were so accustomed to seeing, disappeared altogether. When methanol was added to this mobile phase combination, another peak emerged having a much longer retention time. This suggests that the reagent DNP-NCO may be reacting with the alcohol in the mobile phase, yet further work would be required to confirm.

The US-FDA's views on pharmaceuticals having stereochemistry have become more stringent in the past few years, and a recently released Policy Statement further exemplifies this [28]. Several additional issues must be considered when developing drugs for submission in and IND or NDA to the US-FDA.

4. Conclusions

The literature has a few references on fenfluramine and some elegant work has been performed on both the racemate and on the individual enantiomers, yet this work involved using gas chromatography with electron capture or nitrogen specific detection, where the stability

of the reagent used and/or stereospecificity has been in question.

The prime focus of this study was to develop, validate, and apply an analytical stereospecific HPLC method intended to measure the concentrations of *d*-fenfluramine (administered drug, I) and *d*-norfenfluramine (major metabolite, III), in addition to monitoring *l*-fenfluramine (possible physiologically interconverted compound, II) at the therapeutic plasma concentration range (low ng/ml) and the excreted urine range (ng/ml to μg/ml) after oral administration of enantiopure *d*-fenfluramine. This new methodology has been designed for *in vivo* pharmacokinetic evaluation purposes while monitoring plasma from multi 0-, 5-, 15-, and 30-mg (P.O./B.I.D.) doses and urine from single 30-mg and multi 15-mg doses (P.O.) of enantiopure *d*-fenfluramine hydrochloride.

Approximately 2000 plasma and 1000 urine specimens were analyzed during the clinical trial segments of this study. When we compared the initial validations to that of the subsequent clinical trial data, the resultant statistics, such as inter-assay precision amongst the standards, QAs and calibration curve slopes, were analogous. These data summaries show that this method was dependable, precise, and stereospecific. Additionally, DMLIMS+ allows us to merge and translate clinical specimen data into other computer data base formats (*e.g.* SAS). Final pharmacokinetic statistics and curve fitting are then more easily performed.

Further work is underway in reducing the plasma MQL to less than 10 ng/ml so that one may follow the absorption, distribution, biotransformation, and excretion (ADBE) profile from as low as a single effective oral dose. A similar scheme is also being investigated for monitoring the consistency of the final pharmaceutical product as it goes through production.

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