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Determination of enantiomeric drugs in physiological fluids using on-line solid phase derivatizations and reversed-phase liquid chromatography

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Abstract: A new analytical approach has been developed for the determination of *d,l*-amphetamine in urine using on-line solid phase derivatizations in HPLC–UV/FL. Several other enantiomeric drugs were also investigated using the same method. The method was validated by several experiments, including: (1) kinetic studies for the reaction of each enantiomeric drug with the solid phase chiral reagent; (2) "single blind spiking" experiments; and (3) polarimetry for confirmation of the enantiomeric composition determined by the solid phase diastereomer formation–HPLC–UV/FL method. The resulting diastereomers were well resolved (R_s = 1.05–1.40) under typical reversed-phase HPLC conditions. Enantiomeric contamination at the 1.1% level could be detected. The lowest amount of *d,l*-amphetamine that could be simultaneously derivatized and detected was about 50 ng ml^{-1}. The linearity of the overall measurement was 3–4 orders of magnitude. *d,l*-Amphetamine spiked into urine at different concentration levels and different *d,l*-ratios, only followed by filtration, were directly injected into the on-line solid phase derivatization–HPLC–UV/FL system for quantitation with relative standard deviations 1.8–6.4% and relative errors 0.6–9.8%.

Keywords: *Polymeric chiral reagent; on-line solid phase derivatizations; enantiomeric drugs; kinetic studies of solid phase formation of diastereomers.*

Introduction

Large differences in biological and pharmacological activities between stereoisomers point out the need to accurately assess isomeric purity of pharmaceutical, agricultural, or other chemical substances [1]. Especially important is the determination of the optical purity of enantiomeric drugs. This is very critical, since isomeric impurities may have undesired toxicological, pharmacological, or other side effects on biological systems [2, 3].

It has long been recognized that chromatographic methods could offer distinct advantages over classical techniques in the separation of stereoisomers [4, 5]. There are

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two approaches to the separation of enantiomers by chromatography. In the direct approach, separation of enantiomers is on chiral columns with achiral mobile phases, or on achiral columns with chiral additives in the mobile phase. In the indirect case, separation of diastereomers formed by pre-column derivatization with chiral reagents occurs with achiral columns or mobile phases.

The development of chiral columns has recently been reviewed [6–8], and a number of columns are commercially available [9]. Large separation factors are obtainable in certain cases, and the absolute amounts of enantiomers can be determined without sample work-up/reaction in the direct approach. However, many columns have only moderate efficiency (e.g. protein bonded phases), especially for the separation of some basic drugs, such as amines, and chiral columns are relatively expensive. Chiral mobile phases have also been recently reviewed [10]. These have been made mainly by complexation of chiral ligands in the mobile phase with a transition-metal ion [11], or with a chiral ion-pairing reagent [12]. Enantiomers of neutral, basic, and acidic amino acids, as well as their amides, were resolved and detected at low levels [13]. However, this method required consumption of expensive chiral additives and weaker complexation, in some cases, led to a loss of stereoselectivity, which limited its applications [14]. Separation methods based on an indirect separation of enantiomers by pre-column derivatization with chiral reagents have been reviewed [15–17]. Some of the commonly used reagents for amines are based on isothiocyanates, which give UV-sensitive thiourea derivatives for both primary and secondary amines [18]. The common protein amino acids have been resolved by using 2,3,4,6-tetra-*o*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) [19]. The reactions are selective and proceed to completion under mild conditions. The detectability of enantiomers is enhanced and the chromatographic behaviour of basic compounds is improved as the result of such derivatizations.

We have recently developed a series of novel polymeric achiral and chiral reagents used for the derivatizations of bioorganics in complex matrices, in HPLC–UV–EC–FL detection [20–25]. The polymeric, covalently bonded, achiral/chiral reagents were so designed that the detector sensitive (strong UV/EC/FL response) tagging moieties were activated by functional groups on the polymer backbone, susceptible to nucleophilic attack, thereby leading to fast heterogeneous reactions. There are some significant advantages in performing heterogeneous reactions in comparison with homogeneous ones, especially in the ability to automate and reduce sample work-up and eliminate analyte extraction prior to HPLC. The related theories and method validation were well explained and illustrated in earlier publications [20–25].

This paper describes the approach for the derivatization of enantiomeric drugs using a polymeric FMOC-L-proline reagent under optimized conditions. Also described are the method validation experiments, along with theoretical explanations. It shows a novel and simple method for the determination of *d*/*l*-ratios and the amounts of enantiomeric amphetamine in urine.

Experimental

Apparatus

Measurements were carried out on an apparatus consisting of a Waters model 6000A pump (Waters Chromatography Division, Millipore Corp., Milford, MA, USA), a Rheodyne model 7010 injection valve with 5 and 10 μl sample loops (Rainin Instrument Co., Emeryville, CA, USA), a model SE 120 dual pen recorder (Brown, Boveri and Co.,

Metrawatt/Goerz Division, Vienna, Austria), an EM Science LiChrospher C18 reversed-phase column (Cherry Hill, NJ, USA), 250 × 4.6 mm i.d., 5 μm particle size, a Waters model 480 variable wavelength UV–VIS detector, a Hitachi model F1000 fluorescence spectrophotometer, and a Hitachi model D-2000 ChromatoIntegrator (Hitachi Corp., Naka Works, Mito City, Japan). A Perkin–Elmer model 241 Polarimeter (Perkin–Elmer Corp., Norwalk, CT, USA) was used for the determination of optical rotation and enantiomeric purity of various drugs.

Chemicals, reagents and solvents

The starting polymer, a copolymer of 96% styrene–4% divinylbenzene, was obtained from Fluka Chemical Co. (Buchs, Switzerland). It was 200–400 mesh and 60–90 μm particle diameter. FMOC-L-proline was purchased from Chemical Dynamics Corp. (South Plainfield, NJ, USA). Enantiomeric drugs were obtained from Research Biochemicals, Inc. (Natick, MA, USA), and from the US Food and Drug Administration (Center for Drug Evaluation and Research, Washington, DC). Other chemicals used were obtained from a variety of commercial sources, including: Aldrich Chemical Co. (Milwaukee, WI, USA), J. T. Baker Chemical Co. (Phillipsburg, NJ, USA), Alfa Products (Danvers, MA, USA) and Sigma Chemical Co. (St. Louis, MO, USA). These chemicals were of the highest purity available and were used without further purification, but were checked for purity, at times, before use.

HPLC solvents were obtained from EM Science, Inc. (Cherry Hill, NJ, USA), as their Omnisolv HPLC grade. All HPLC solvents were used after filtration through a 0.45 μm solvent filter (GVWP, Millipore Corp., Bedford, MA, USA) and degassed under vacuum with stirring.

Synthesis of polymeric FMOC-L-proline reagent

FMOC-L-proline, 0.6 g (1.8 mmol), was dissolved in 10 ml methylene chloride. The solution was cooled to 0°C with ice water and 1.8 mmol of dicyclohexylcarbodiimide (DCC) was added. After 30 min at 0°C, the mixture was filtered to remove the precipitated excess reagents. The filtrate was poured into a 100 ml flask containing 1 g of polymer-bonded 4-hydroxy-3-nitrobenzophenone [28]. Pyridine (0.5 ml) was added and the reaction mixture was stirred at ambient temperature for 1 h. The polymer was filtered and washed with hexane (3 × 50 ml), methylene chloride (3 × 50 ml), and acetonitrile (3 × 100 ml). The polymeric chiral reagent was obtained as a light brown solid (1.1 g).

Determination of the optical purity of FMOC-L-proline

FMOC-L-proline was dissolved in dimethylformamide (DMF) to a concentration of 10 parts per thousand (mg ml^{-1}). The optical rotation of the reagent was measured using a polarimeter under standard conditions. At a constant temperature, 20°C, a sodium lamp with the D line at 589 nm was used as a polarized light source. Three measurements were taken against a solvent blank. The instrument was calibrated using a USP reference standard, *R*-(*S*)-amphetamine, with 99.99% optical purity (US Pharmacopeia, Philadelphia, PA).

Procedures for on-line derivatization reactions

Stainless steel reaction columns (27 × 20 mm i.d.) were made in this laboratory. The reaction column was dry-packed by directly filling the reactor with the reagent with

tapping several times. Using a Rheodyne Model 7060 injector as a switching valve, the reaction column was connected to the loop position on the valve. The reaction column was placed into a constant temperature water bath (60°C). The sample solution containing enantiomeric drugs (10 μl) was injected and the switching valve was switched to the by-pass position at the correct time (*c.* 6 s). The analyte was held within the reaction column for a specific amount of time (5 min), and the valve was then switched back to flush the derivative from the reaction column into the separation column. The final optimized on-line condition for the derivatization of enantiomeric drugs was reaction at 60°C for 5 min, mobile phase 40–48% ACN–H_2O, flow rate 1.5 ml min^{-1}, separation column LiChrospher C_{18}, 5 μm, 250 × 4.0 mm i.d., UV 265 nm, FL 265/315 nm.

Spiking of d,l-amphetamine into urine

Fresh urine was neutralized with 1 N NaOH to pH 10. Aliquots of the urine sample (10 ml) were then spiked at four different, known concentration levels of each *d*- and *l*-amphetamine standard, and with four different *d*/*l*-ratios. The spiked urine samples were then filtered and analysed as urine taken from someone who had ingested *d,l*-amphetamine.

Standard addition analysis

Four different concentrations of *d*- and *l*-amphetamine (32.5 and 65 μg ml^{-1} *d*-form; and 35.5 and 71 μg ml^{-1} *l*-form) were prepared and added to each spiked urine sample. To each spiked urine sample was added two different, known concentrations of *d*- and *l*-amphetamine. Three injections were made for each sample with or without standard addition. Three-point calibration plots were then constructed for the determination of *d*/*l*-ratios and absolute levels/amounts of *d*- and *l*-amphetamine spiked in the individual urine samples.

Results and Discussion

Solid phase derivatizations of enantiomeric amphetamine

The synthetic procedures, schemes, and the characterization of the polymeric FMOC-L-proline reagent have been described in our previous publication [25]. The scheme for the derivatization of enantiomeric amphetamine with the polymeric FMOC-L-proline reagent is given in Fig. 1. The nucleophilic substitutions at the carbonyl carbon attached to the polymer bonded 4-hydroxy-3-nitrophenone linkage, are extremely fast due to the FMOC-L-proline tag being activated by electron withdrawing groups, *o*-nitro and *p*-carbonyl, on the polymer backbone. In the process, the polymer bonded 4-hydroxy-3-nitrophenone anion becomes a good leaving group due to the resonance effect stabilizing this ionic species. Other strong nucleophiles in the samples, such as OH^-, would also react with, or hydrolyse the polymeric reagent. However, the hydrolysis product(s) were eluted in the solvent front or as earlier eluted peaks, totally separated from the derivatives of interest under the reversed-phase conditions [23, 24].

Optical purity of the polymeric FMOC-L-proline reagent

The indirect chiral separation mode requires a highly pure chiral reagent to react completely with enantiomeric substrates that may be impure (<100% optical purity). An error in the determination of both optical and chemical purities of the enantiomers will

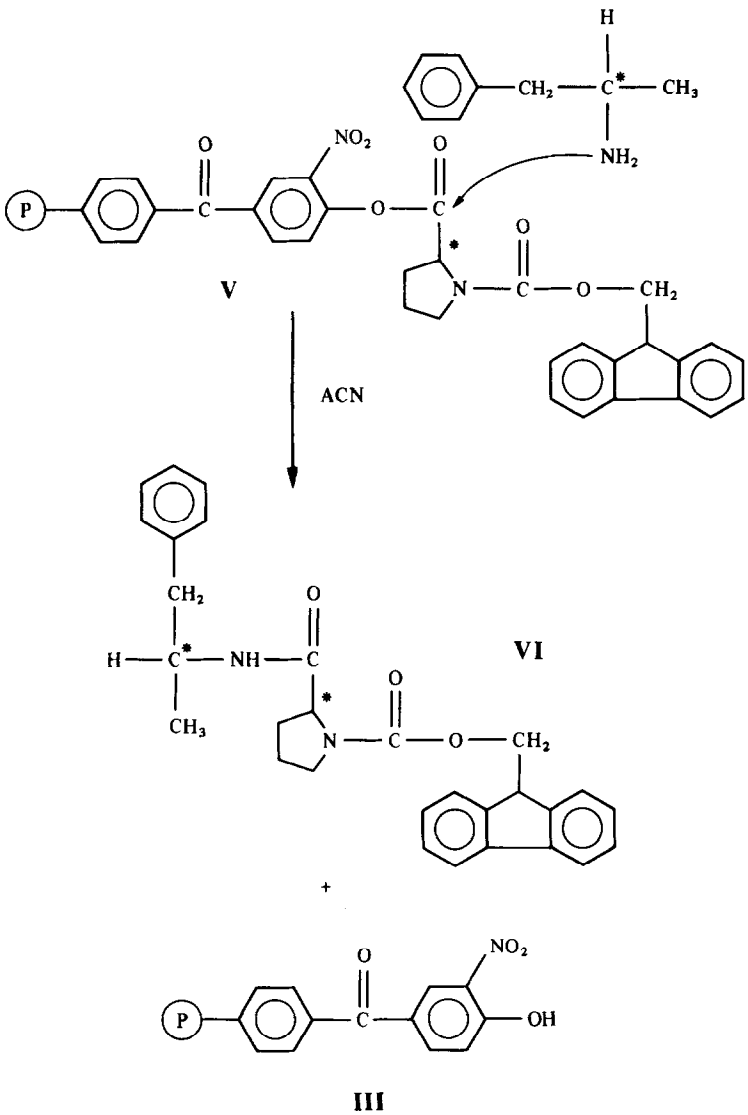

Figure 1
Reaction of FMOC-L-proline chiral reagent with *d,l*-amphetamine.

be generated if the chiral reagent is not sufficiently optically pure, since four possible stereoisomers can be formed after the reaction. However, only two diastereomers from the four stereoisomers possible, can be chromatographically separated using a conventional (achiral) reversed-phase column [26]. It is stated in the literature [27, 28], and from our own experience [25], that there has been no racemization during the solid phase synthesis nor during the derivatizations using analogous chiral reagent(s). The only possible source of the enantiomeric contamination from the solid phase chiral reagent would therefore be from any enantiomeric (optical) impurities in the starting chiral reagent (i.e. FMOC-L-proline). The optical purity of the chiral tag, FMOC-L-

proline, was therefore checked using a polarimeter, under standard measurement conditions. A USP reference standard, a highly optically pure compound (99.99%), *d*-(*S*)-amphetamine, was used to calibrate the polarimeter. The specific rotation of FMOC-L-proline ($[\partial]_D^{20}$) was $-33.0° \pm 0.5°$. By comparing this number with that in the literature [29], and by inclusion of any measurement errors, the measured optical purity for the FMOC-L-proline was 99.2 ± 0.2%. Although there was still a small amount of the *d*-form impurity (*c.* 0.8%) existing in the polymeric FMOC-L-proline reagent, errors caused by this optical impurity would be insignificant. This was actually shown by an experiment, in which a USP reference standard, *d*-(*S*)-amphetamine, at a relatively high concentration level (100 μg ml^{-1}), was derivatized under conditions such that the *d,l*-amphetamine diastereomers could be well resolved. If the 0.8% optical impurity was significant, two diastereomer peaks should have appeared in the chromatogram. One peak was possibly from *l–d* and *d–l* diastereomers (enantiomerically related), and the other peak from *d–d* and *l–l* diastereomers (enantiomerically related) when a conventional reversed-phase (achiral) HPLC column was used. However, only a single peak (*l–d* diastereomer, *l*-form tag combined with *d*-form amphetamine) was realized (Fig. 2), suggesting that the polymeric reagent was optically pure enough and the method was suitable for chiral recognition.

Determination of enantiomeric contamination of l-amphetamine

Using the same conditions described above, *l*-amphetamine was prepared in basic solution (pH 10, 100 μg ml^{-1}), and was directly injected into the on-line, pre-column

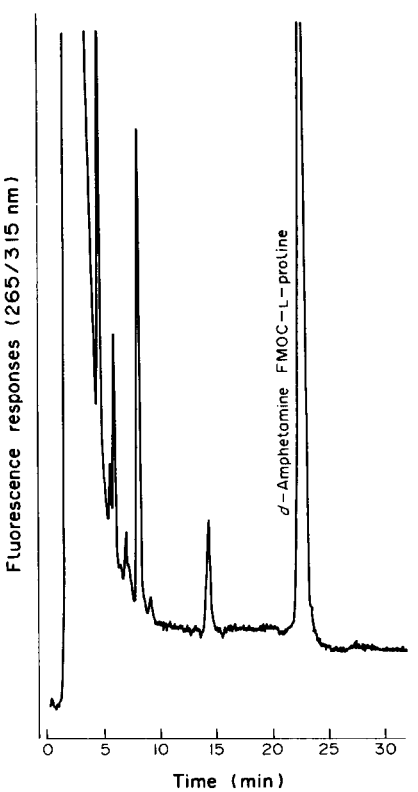

Figure 2
Chromatogram of highly pure *d*-amphetamine derivative.

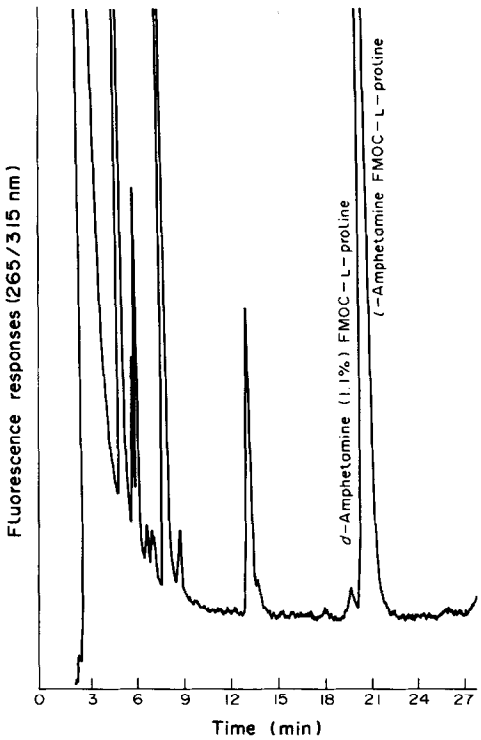

Figure 3
Determination of the smallest amount of one enantiomer in the presence of the other.

derivatization–HPLC–UV/FL system. The injections were repeated three times at the same concentration level. A small *d*-amphetamine diastereomer peak was realized (Fig. 3). The *d*-form contamination in the *l*-form major component was 1.1 ± 0.5 ($n = 3$), calculated from the peak area ratio using an integrator. This may have resulted from the synthetic process used to prepare the original drug.

Optimization of reaction and detection conditions

The basic purposes for the chiral derivatization of enantiomeric drugs were to improve the separation behaviour, and to enhance the selectivity and sensitivity. It was desirable to: (1) have high percent conversions of enantiomers to diastereomers, to ensure high sensitivity of the method in the determination of enantiomeric drugs at trace levels; (2) have diastereomers well resolved with good (high) column efficiency maintained (high theoretical plate number and low band broadening); and (3) have equal UV/FL detector responses for both diastereomers. These ideal goals could be achieved by optimizing the system conditions.

It has been shown that more than 80% derivatizations for the primary amines were obtained using an analogous achiral polymeric reagent, on-line, in pre-column derivatizations at 60°C, stop-flow, for 5 min. A <100% conversion might have been caused by the partial decomposition of the derivative(s) under these conditions [23, 24]. Having a primary amino functional group in the amphetamine molecule, and using the same polymeric backbone, but with a different chiral tag, a similar loading of the tag/g reagent (compared with the achiral polymeric reagent), similar percent derivatizations of *d,l*-amphetamine should have been obtained under the same reaction conditions. The pre-column, solid phase reactor with a switching valve was again used in the current

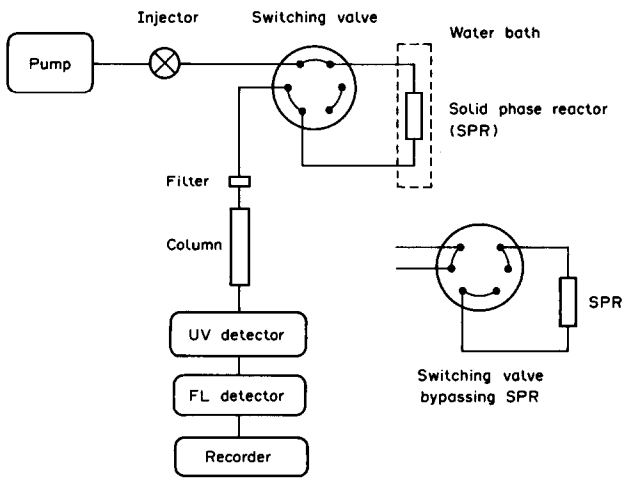

Figure 4
Instrumentation for on-line, pre-column solid phase derivatization HPLC–UV/FL detection.

study (Fig. 4), in view of its previously reliable performance. A detector response plateau for the *d,l*-amphetamine diastereomers was obtained after a 5 min reaction at 60°C. This did not ensure 100% conversions (derivatizations); however, it suggested that the highest percent derivatizations were obtained under these conditions.

Acetonitrile (ACN) was again used as the derivatization solvent, which consistently provided the highest percent derivatizations of all solvents, including: methanol, dioxane, hexane, and tetrahydrofuran (THF). It also acted as a typical organic modifier for good separations in the reversed-phase HPLC mode. The amine substrates can also be derivatized with this polymeric reagent in ACN–aqueous mixture. *d,l*-Amphetamine was, therefore, prepared in 50:50 ACN–H_2O (pH 10). This sample solution (10 μl) was directly injected into the on-line solid phase derivatization HPLC system for chiral derivatization. The baseline separation of the resulting diastereomerically related derivatives was achieved using 48% ACN–H_2O (Fig. 5).

The determined maximum injection volume that can be held in the reaction column was 20 μl. However, a 10 μl injection volume was chosen to ensure that the total injection volume was in the reaction column. Having a fixed injection volume, concentration of the substrates was important to maintain linearity of the calibration plots.

The UV and FL spectra of *d,l*-amphetamine FMOC-L-proline diastereomers in 50:50 ACN–H_2O, were run using an on-line fluorescence spectrometer/detector. The optimum wavelengths were 265 nm for UV absorbance, and 265/315 nm for the fluorescence excitation and emission, respectively.

Kinetic studies of reactions of d,l-amphetamine with the polymeric chiral reagent

Theoretically, enantiomers, due to their different chemical activities, may have potential (often real) differences in their reaction rates, when they react with another chiral compound [31]. To form the correct amounts of *d*- and *l*-diastereomers from enantiomers, a completion (100%) reaction is usually required in any indirect chiral

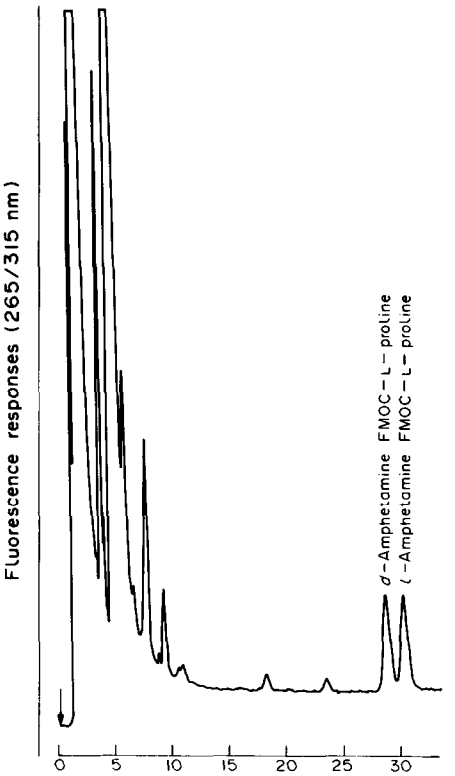

Figure 5
Chromatogram of *d,l*-amphetamine.

separation method, HPLC or otherwise. It would be strong evidence for the partial validation of our method, if the *d/l*-ratios of diastereomers obtained after the reaction, were equal to that of the enantiomers. Kinetic studies, therefore, were designed with this goal in mind.

A racemic mixture of *d,l*-amphetamine was prepared in 50:50 ACN–H_2O. This solution was injected into the on-line, pre-column derivatization HPLC–UV/FL system. Under optimized conditions, each injection of the racemic solution was allowed to reside in the reactor at reaction times of 10, 20, 30, 60, 180, 300, and 600 s. This was done using a switching valve (Experimental). The peak areas of both *d*- and *l*-diastereomers were integrated at these different reaction times. Plots of the peak areas of *d*- and *l*-diastereomers versus reaction times were then constructed (Fig. 6), using three injections for each data point. Within experimental error, the peak areas of *d*- and *l*-diastereomers were the same. There are several explanations: (1) the chemical reactivities of these *d*- and *l*-amphetamines were not significantly different; and (2) the chiral tag was highly activated, leading to fast reactions for both *d*- and *l*-enantiomers, which offset any small differences in their overall reactivities (specific rates of reaction were equal).

Based on the same kinetic study experiments, a plot of peak area ratios of *d*- and *l*-amphetamine diastereomers versus reaction times was also constructed. The *d/l*-ratio was around unity at different reaction times, with an acceptable experimental error, which again suggested that the same reaction rates for *d*- and *l*-amphetamine were obtained from 10 to 600 s. All data from the kinetic studies, not only provided a possible way to

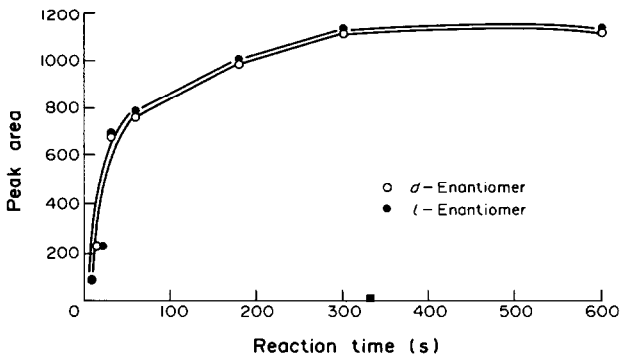

Figure 6
Peak area of *d,l*-amphetamine diastereomers versus reaction time.

validate the method, but also suggested that, with these particular solid phase reactions, diastereomer formation does not have to be 100% for chiral recognition/quantitation to be accurate. This was true as long as reactions, resolutions, and detector signals were reproducible. This meant that great savings in reaction times could be obtained, together with other analytical advantages realized.

Separation parameters for d,l-amphetamine diastereomers

The separation behaviour of the diastereomeric amphetamine derivatives was investigated as a part of method optimization. The *d*-amphetamine diastereomer was found to elute before the *l*-amphetamine diastereomer, and this may have been due to the different solubilities of these diastereomers, or caused just by different physical properties. The adsorption/partition interactions of an analyte within the reversed phase will increase with a decreasing composition of the organic modifier in the mobile phase. The *d*- and *l*-diastereomers would then be better separated. However, this would result in increasing bandwidth and longer analysis times per run. Under optimized separation conditions, typical separation parameters were measured with three different *d*/*l*-ratios.

Selectivity factors (α) were larger than unity, theoretical plate numbers (N) were greater than 8500, and resolution (R_s) values were all above 1.21 (Table 1). These data suggested that high column efficiencies and baseline separations could be obtained under these optimized conditions. All of these calculations were done using formulae described in the literature [32].

Trace determination of d,l-amphetamine

It would become more meaningful, useful, and practical, especially in real-world sample applications, to demonstrate the smallest amounts of enantiomers that could be simultaneously derivatized and detected/quantitated using this final method, rather than just reporting the detection limits derived from the diastereomers alone. Equal amounts of *d*- and *l*-amphetamine were mixed to form a 50:50 mixture with different concentrations. A blank (solvent, 50% ACN–H_2O, 10 μl) was injected into the on-line derivatization, pre-column HPLC system, followed by injections (n = 3) of the sample solutions, all under optimized conditions. After comparing the chromatograms of the samples with those for the blank, a pair of well separated *d*- and *l*-amphetamine

Table 1
Separation parameters of *d,l*-amphetamine diastereomers*

d/l-ratio	α†	*N*‡	R_s§
1:1	1.08(0.02)‖	8846(126)	1.23(0.02)
2:1	1.07(0.03)	8788(130)	1.22(0.01)
1:2	1.06(0.02)	8687(110)	1.21(0.01)

*Reaction detection conditions: 60°C, 5 min, 7.2–14.5 ppm *d/l*-amphetamine (pH 10), 10 μl injection, 48% ACN–H_2O, 1.5 ml min^{-1}.
†Selectivity factor, $\alpha = k_2'/k_1'$.
‡Theoretical plates, $N = 5.54\ (t_r/W_{0.5})^2$.
§Resolution, $R_s = [N^{1/2}/4 \times (\partial - 1)/\partial \times (k'/(1 + k'))]$.
‖Average (standard deviation, $n = 3$).

diastereomer peaks having equal detector responses (same peak areas), were realized at a concentration level of 50 ng ml^{-1} (0.5 ± 0.1 ng) for each enantiomer. The signal-to-noise (S/N) ratio was 3:1 (Fig. 7). The smallest amount of *d*-amphetamine in the presence of a large amount of *l*-amphetamine (*d*-form–*l*-form = 1.99), was 1.1 ± 0.8 ng ($n = 3$) after normalizing S/N to 3:1 (Fig. 3). Considering enantiomeric amphetamines as natively poor chromophores, a significant improvement in detectability of these enantiomers, combined with chiral separation, was achieved as the result of the on-line, solid phase derivatization–HPLC–UV/FL. The normally occurring enantiomeric drugs in physiological fluids occur at ppm levels or above [33]. The sensitivity of the method is more than adequate for chiral recognition and quantitation in typical complex matrices.

Calibration plots of d,l-amphetamine diastereomers

Starting from the lowest detectable amounts of racemic amphetamine, a series of racemic amphetamine solutions at different concentrations were prepared (0.05, 0.5, 5, 50, 100, 200, 300, and 500 μg ml^{-1}). At least three injections of the sample at different concentration levels, were made into one reaction column, under optimized conditions, for on-line solid phase derivatizations. Calibration curves of racemic amphetamine diastereomers were then constructed. Non-linear responses were realized after derivatizing samples containing 300 μg ml^{-1} *d,l*-amphetamine. This might be caused by the gradual depletion of the FMOC-L-proline tag by the substrate. The linearity of the plots were 3–4 orders of magnitude, suggesting a relatively wide linear dynamic range using one reactor containing the chiral reagent. Valid determinations possible before observation of diminished response depends on the amount of substrate to be derivatized, the loading and the amount of the polymeric reagent to be used. For example, more than 50 on-line derivatizations of 0.2 μg simple propylamine with the analogous achiral polymeric reagent (0.8 meq g^{-1}, 65 mg), could be obtained without changing peak area/height [24].

Separation of other enantiomeric drugs

Separations of other enantiomeric drugs, including norephedrine, pseudoephedrine and DOB [1-(2,5-dimethoxy-4-bromophenyl)-2-aminopropane], were successful using on-line, pre-column solid phase derivatizations under optimized conditions. Kinetic studies for these drugs were also performed using the above described approach. In all cases, the *d*- and *l*-enantiomers had similar reaction rates, and the *d/l*-ratio was constant

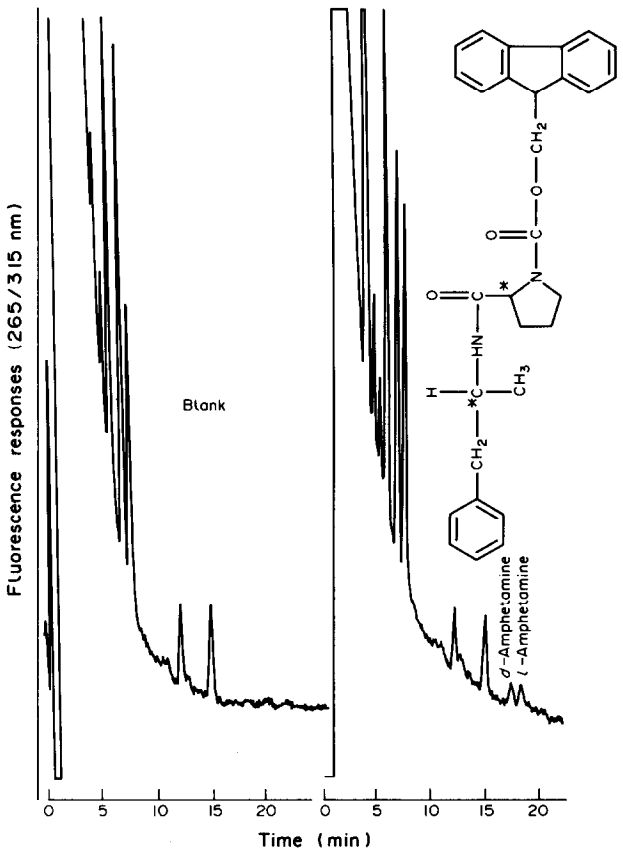

Figure 7
Chromatogram of the smallest amount of *d,l*-amphetamine being derivatized and detected via on-line solid phase derivatization.

with varying reaction times. The method was versatile, and was further validated by the data obtained from these additional drug experiments. The separation parameters for these enantiomeric drugs are given in Table 2.

Determination of optical rotation of racemic drugs using polarimetry

The *d/l*-ratios determined by this derivatization–HPLC method were all racemic (50:50). These results were confirmed by polarimetry. The degree of optical rotation of any truly racemic enantiomers should be zero, because the two optically active components rotate polarized light to the same extent, but in opposite directions [34]. Thus, the final degree of optical rotation will be cancelled out. The optical rotation of each racemic drug sample was measured three times using a polarimeter under standard operating conditions (20°C, sodium lamp, D line, 289 nm). Within the measurement error, the measured optical rotations of all racemic drugs were zero, which were all consistent with the expected values, initially suggested from the derivatization–HPLC–UV/FL data. This experiment provided yet another approach for overall method validation.

Table 2
Separation parameters of enantiomeric drugs

Drug	*d/l*-ratio	α	*N*	R_s
DOB	1:1	1.08	9140	1.40
Norephedrine	1:1	1.05	8188	1.15
Pseudoephedrine	1:1	1.06	8789	1.21

Conditions: *d,l*-enantiomeric mixture (50:50), 10–20 ppm, in 50% ACN–H_2O (pH 10), 10 μl injections, three injections for each drug, 60°C, 5 min stop flow, on-line derivatizations. LiChrospher C18, 5 μm, 250 × 4.0 mm i.d., 40–50% ACN–H_2O, 1.5 ml min^{-1}, UV 265 nm, FL 265/315 nm.

Determination of enantiomeric amphetamines in urine

It would be attractive if the analytical method could be used for the determination of enantiomeric drugs in complex biological samples. Therefore the feasibility of this method was investigated for use in determination of enantiomers in physiological fluids, such as urine. This study was performed in a "single blind spiking" manner.

Currently, such applications usually involve tedious sample preparation/extraction, leading to time consuming steps and possible loss of analytes at trace levels [35, 36]. The approach described here avoids all types of sample preparation, or any solvent and solid phase extraction, but rather uses just filtration and direct injection. It is perhaps the *simplest* of all possible analytical methods for *d,l*-amphetamine and many other drugs commonly found in physiological fluids. *d,l*-Amphetamine was spiked into urine in different *d/l*-ratios and concentrations. The spiked urine samples were determined with a minimum of sample work-up, using on-line, pre-column solid phase derivatization, reversed-phase separation, and final UV/FL detection. Each urine sample was directly injected (10 μl) three times for quantitation under optimized on-line reaction conditions (60°C, 5 min). Symmetric peak shape, reproducibility, baseline stability, and baseline separations were obtained with different *d/l*-ratios and concentrations (Fig. 8). Standard addition methods, combined with three point plots, were used for all final quantitations. The results are indicated in Table 3, again showing overall good precision and accuracy. The relative standard deviations (%RSD) were 1.8–6.4% and the percent relative errors (%RE) were 0.6–9.8%, all of which were compatible with similar precision results reported for alternative methods [33, 35, 36].

Conclusions

We have demonstrated a novel method for the determination of enantiomeric drugs in physiological fluids, such as urine. Several approaches were designed and investigated for overall method validation. Reactions could be successfully performed both on-line and off-line, under relatively mild reaction conditions, especially using short reaction times. On-line derivatizations using polymeric chiral reagents have proven compatible with conventional, isocratic reversed-phase HPLC solvents, flow rates, and reasonable reaction temperatures. Kinetic studies have also provided valuable information for these heterogeneous reactions of enantiomeric drugs. It is worthwhile to note that there may exist some *d,l* pairs which may not have identical reaction rates with this polymeric chiral reagent. This particular polymeric chiral reagent has exhibited good thermal and aqueous stability, high percent derivatizations, low detection limits, few interferences in

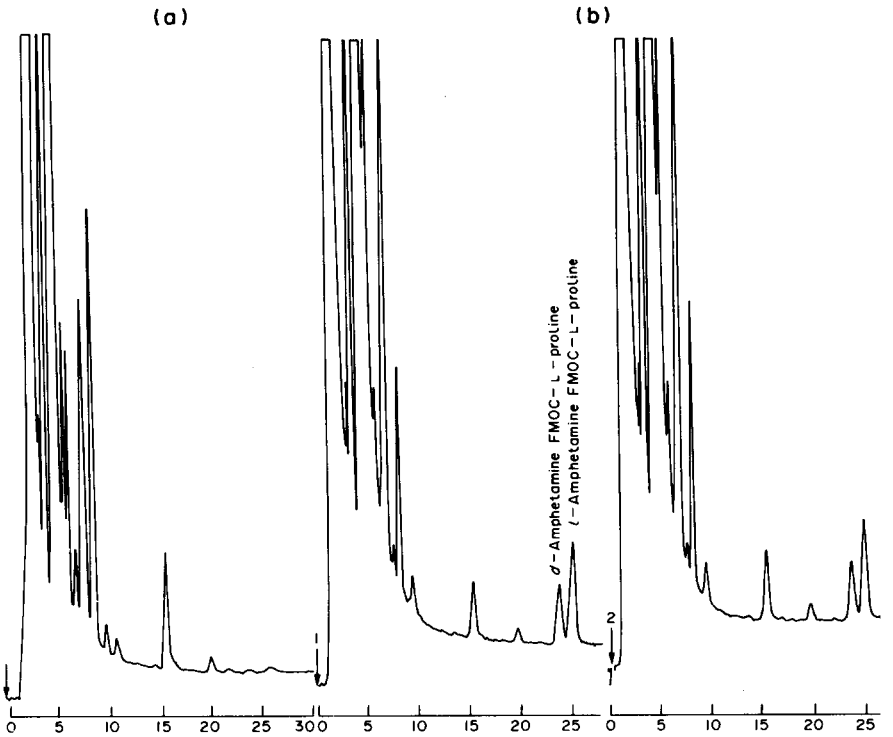

Figure 8
(a) Urine blank; (b) repeated injections of spiked urine sample with *d*,*l*-amphetamine via on-line solid derivatizations.

Table 3
Determination of *d*,*l*-amphetamine diastereomers spiked into urine (single blind experiment)*

Sample	Spiked (ppm)	Found	%RSD†	%RE‡
No. 1				
d-Form	56.5	55.7 (1.0)§	1.8	1.4
l-Form	30.9	32.6 (0.9)	2.3	5.5
No. 2				
d-Form	6.31	6.93(0.41)	5.9	9.8
l-Form	13.8	14.0 (0.50)	3.5	1.4
No. 3				
d-Form	9.47	9.53(0.61)	6.4	0.6
l-Form	10.34	10.8 (0.50)	4.6	4.4
No. 4				
d-Form	11.6	12.0 (0.4)	3.3	3.4
l-Form	63.4	60.8 (2.8)	4.6	4.1

*Reaction detection conditions: 60°C, 5 min, 48% ACN–H_2O, 1.5 ml min^{-1} flow rate, UV 265 nm, FL 265/315 nm.
†Relative standard deviation, RSD = $s/X \times 100$.
‡Relative error, RE = (value found − true value)/true value × 100.
§Average (standard deviation, $n = 3$).

the HPLC–UV/FL chromatograms, and fast chiral recognition/quantitation of enantiomeric drugs. The major limitations may be the difficulties in derivatizing secondary amino functional groups and the requirement for high optical purity of the chiral reagent.

Finally, the method has been shown practical and valid by its application to urine samples. After filtration, there was no further requirement for additional sample work-up or drug extraction. It is believed that this is the simplest, while still accurate and precise, and fully automatable, method for the simultaneous determination of both optical and chemical purities of enantiomeric drugs in complex sample/biological matrices.

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