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# Determination of D-fenfluramine, D-norfenfluramine and fluoxetine in plasma, brain tissue and brain microdialysate using high-performance liquid chromatography after precolumn derivatization with dansyl chloride

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**Abstract**

A HPLC method is described for the simultaneous determination of D-fenfluramine (FEN), D-norfenfluramine (NF) and fluoxetine (FLX) using fluorometric detection after precolumn derivatization with dansyl-chloride. The method has limits of quantitation of 200 fmol for FEN and NF, 500 fmol for FLX in brain microdialysate, and 1 pmol for NF and FEN, and 2 pmol for FLX in plasma. Brain tissue standards were linear between 5 and 200 pmol/mg for all three compounds. The inter-assay variability (relative standard deviation) was 6.6%, 6.9% and 9.3% for FEN, 4.6%, 3.7% and 7.9% for NF and 10.4%, 4.9% and 12.2% for FLX, for brain microdialysate (2 pmol/μl), plasma (2 pmol/ μl) and brain tissue (50 pmol/mg), respectively. Intra-assay variability was always lower, typically several times lower than inter-assay variability. Extraction recovery was 108% and 48% for FEN, 105% and 78% for NF and 94% and 45% for FLX, in plasma (2 pmol/μl) and brain tissue (5 pmol/mg), respectively. Due to the stability of the dansyl-chloride derivatives this method is well suited for an autoinjector after manual derivatization with dansyl chloride at room temperature for 4 h.

*Keywords:* Fenfluramine; Norfenfluramine; Fluoxetine

## 1. Introduction

D-Fenfluramine (FEN, $M_r$=231) and fluoxetine (FLX, $M_r$=309) are cyclic secondary amines which act primarily as serotonin re-uptake inhibitors at lower concentrations and as serotonin releasers at higher concentrations [1,2]. FENs major metabolite D-norfenfluramine (NF, $M_r$=203) is a primary amine that preferably acts as a serotonin releaser [3]. FEN is used as an anti-obesity drug [1] and FLX (Prozac) is used as an antidepressant [4]. Considerable interest has existed for more than a decade in the pharmacokinetics of these compounds due to their clinical application and their use as pharmacological tools.

Gas chromatographic methods are most commonly used [5–10] for quantitation of FEN and NF, with minimum quantifiable amounts typically at 2–2.5 ng/ml [6,10]. Like an earlier GC method, [8] a recently published HPLC method utilizing UV detection [11] is capable of separating optical isomers of fenfluramine; however, its sensitivity (minimum

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quantitation limit in plasma 10 ng/ml) was lower than the (nonstereospecific) GC methods mentioned above. Several GC methods [12–14] and a considerable number of HPLC methods [15–20] have been published for FLX (and its major metabolite norfluoxetine). The minimum quantifiable amounts varied between 5 and 20 ng/ml plasma for the GC-methods [12–14]. All but one of the technically less difficult HPLC methods were based on UV detection and their sensitivity ranged from 6 to 25 ng/ml plasma [15,16,18–21]. According to our previous experience with the analysis of amphetamine [22] we expected that a HPLC method with fluorescent detection would be more sensitive and in fact one paper describes the use of fluorescent detection at a quantifiable concentration of 1 ng/ml plasma for FLX [17].

The previously published methods usually aimed at analyzing FEN or FLX and metabolites in human samples with plenty of sample volume (e.g., 1–10 ml of plasma) available. In contrast, our analytical problem was to quantitate FEN and NF in much smaller samples from rats, specifically in brain microdialysate (10 μl), brain tissue (50–100 mg) and plasma (100 μl). FLX was used as an internal standard. Therefore, we were seeking a method which was technically less difficult than GC, more sensitive than HPLC–UV and suitable for an autoinjector.

For many years, 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride, Dns-Cl) has been used as a derivatizing agent for primary and secondary amines with a high fluorescent yield [23] and with excellent stability of the derivatives [19,24]. A major obstacle in using Dns-Cl is the occurrence of a large system peak, supposedly due to Dns-Cl hydrolysis [25] or unreacted Dns-Cl. It has been proposed to cleanup the chromatogram by either using the minimum amount of Dns-Cl necessary [25] or by reacting excess Dns-Cl with added glycine and extracting the dansylglycine with a $C_{18}$ Sep-Pak cartridge [24]. Due to our small sample volumes, we considered an additional extraction step with $C_{18}$ Sep-Pak cartridges as inappropriate and neither the reduction of the amount of Dns-Cl nor modifications of the gradient program were sufficient to avoid interference of unidentified peak(s) with the detection of NF. However, we successfully explored the applicability of a strong anionic resin for clean-up purposes. In the following we describe a HPLC-method with fluorescent detection for quantitating dansylated FEN, NF and FLX after removing excess Dns-Cl with resin.

## 2. Experimental

### 2.1. Chemicals

D-Fenfluramine hydrochloride was obtained from Research Biochemicals International (Natick, MA, USA), fluoxetine hydrochloride was obtained from Lilly (Indianapolis, IN, USA), D-methamphetamine sulfate and Dns-Cl were obtained from Sigma (St. Louis, MO, USA). D-Norfenfluramine was a gift from S. Lorens (Loyola University, Maywood, IL, USA). Acetonitrile and ethyl acetate were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sodium tetraborate decahydrate was obtained from Fluka (Buchs, Switzerland) and sodium heparin (1000 USP units/ml) from Solopak Laboratories (Elk Grove Village, IL, USA). Macro-Prep 50 Q strong anion-exchange support (resin) was purchased from Bio-Rad (Richmond, VA, USA). Purified water from a Milli-Q water system (Millipore, Marlborough, MA, USA) was used for the preparation of buffers and standards.

### 2.2. Equipment

A Waters HPLC system (Waters Associates, Milford, MA, USA) was used and included the following components: Two Model 510 pumps controlled by a Model 680 gradient controller, a Model 474 scanning fluorescence detector and a Model 746 data module integrator. Chromatographic separation was performed on a Supelco 5 μm LC-18 150×4.6 mm I.D. analytical column (Supelco, Bellefonte, PA, USA). A C-130B 20×2 mm I.D. guard column (Upchurch Scientific, Oak Harbor, WA, USA), was packed with Supelco No. 5-8294 LC18 40 μm pellicular packing (Supelco). This HPLC system was connected to a CMA/200 autoinjector (Carnegie Medicine, Stockholm, Sweden). For centrifugation of blood and sonicated tissue a Hermle Z 360 K

centrifuge (Gosheim, Germany) was used. The resin was sedimented with an Eppendorf 5412 centrifuge.

### 2.3. Collection of body fluids and brain samples

Microdialysis samples were collected as described elsewhere [26]. The artificial cerebrospinal fluid consisted of 145 m*M* NaCl, 1.5 m*M* KCl, 1.5 m*M* $MgCl_2$, 1.25 m*M* $CaCl_2$, 10 m*M* glucose and 1.5 m*M* $K_2HPO_4$, adjusted to pH 7.0 with HCl. This fluid was pumped through a CMA/12 microdialysis probe (2 mm probe tip length) at a flow-rate of 1 μl/min. For the collection of plasma, rats were decapitated and trunk blood was collected into glass tubes containing 100 μl of sodium heparin. Blood samples were centrifuged under refrigeration for 10 min at 18 000 *g*. Brains were quickly removed from the skull and dissected on a chilled glass plate. Samples of brain tissue and blood were also collected from untreated animals for use as blanks or to be spiked with standards. All samples were stored at −80°C until analysis.

### 2.4. Preparation of Dns-Cl reagent

For fluorescence detection, the Dns-Cl reagent was prepared as described by Bravo et al. [25] with the modification that Dns-Cl was not dissolved in acetone but rather dissolved in acetonitrile (4.5 mg/10 ml) because it has been shown that the reaction in acetonitrile produces a higher yield of fluorescence [24]. The Dns-Cl solution stored under nitrogen in a glass vial at 0°C was stable for at least one month [25].

### 2.5. Sample preparation and extraction procedures

Microdialysis samples (10 μl) were injected directly after 4 h of derivatization with 90 μl of Dns-Cl and resin cleanup (see Section 2.6).

Plasma samples were extracted under the following basic conditions: 200 μl of 0.1 *M* borate buffer (adjusted to pH 10.6 with sodium hydroxide) and 400 μl of ethyl acetate were added to an aliquot of 90 μl of plasma plus 10 μl of an aqueous solution containing the internal standard (20 pmol/μl). The mixture was vortex-mixed for 2 min and placed on ice. After 10 min an additional 400 μl of ethyl acetate and 400 μl of water were added to the mixture followed by brief vortex-mixing to further facilitate extraction. Centrifugation for 10 min at 18 000 *g* and 4°C was used to separate the organic and aqueous phases. Then, 400 μl of the organic supernatant was transferred to a 1.5 ml polypropylene vial and dried under a stream of nitrogen at 45°C. The analytes were reconstituted in 10 μl of 0.1 *M* potassium phosphate (pH 2.5). After adding 7–8 mg of solid sodium bicarbonate the samples were reacted with 90 μl of Dns-Cl (see Section 2.6).

Brain samples were weighed and diluted with 9 volumes (i.e., nine-fold of the mg brain weight in μl) of 0.1 *M* borate buffer (pH 10.6) containing the internal standard (5 pmol/μl FLX). After 20 s of ultrasonication, the samples were centrifuged for 10 min at 18 000 *g* and 4°C. The analytes were extracted from 50 μl of the supernatant using the same procedure as for plasma.

### 2.6. Derivatization and resin-cleanup

Sodium bicarbonate (6–7 mg for microdialysis samples, 7–8 mg for plasma and brain extracts) was weighed into the reaction tube either after the extracted sample was dried and reconstituted in potassium phosphate (plasma and brain tissue) or before the sample was added (microdialysate). Then, 90 μl of the Dns-Cl solution was added. The tube was closed, vortexed for 10 s, and maintained at room temperature in the dark. Four hours later, a 75-μl aliquot of the Dns-Cl reactant was transferred to a tube containing 75 μl of sodium bicarbonate (0.1 *M*, adjusted to pH 10) and approximately 5–7 mg of Macro-Prep 50 Q strong anion-exchange resin were added which previously had been equilibrated with sodium bicarbonate (0.1 *M*, adjusted to pH 10). The mixture was vortexed for 2 min and spun down. The resin trapped the majority of the excess Dns-Cl removing the interfering peaks from the chromatogram. The strong anion-exchange resin probably binds charged compounds because of their polarity. The "excessively large system peak" is supposedly due to dansyl chloride hydrolysis [25]. Thus, the hydroxylated Dns-Cl will be trapped by the strong anionic resin. This idea is also supported by our observation that serotonin (5-hydroxytryptamine) which carries a hydroxyl group disappears after

Table 1
Chromatographic gradient conditions for HPLC determination of Dns-Cl derivatives

| Time (min) | Buffer A (%) | Buffer B (%) |
|---|---|---|
| Initial (0) | 100 | 0 |
| 0–6 | 100 | 0 |
| 7 | 20 | 80 |
| 12 | 20 | 80 |
| 12.5 | 0 | 100 |
| 18 | 0 | 100 |
| 19 | 100 | 0 |
| 30 | 100 | 0 |

resin-cleanup, but can be detected in samples without resin-cleanup. From the supernatant, a 105 μl aliquot of the solution was transferred to an autoinjector vial which then was capped, sealed with a crimper, and placed into the autoinjector sample tray.

### 2.7. Chromatographic conditions

A linear program (Table 1) was run at room temperature with mobile phase A consisting of 50% potassium phosphate buffer (25 m*M*, adjusted to pH 4.5 with phosphoric acid) and 50% acetonitrile, mobile phase B consisting of 25% of the same potassium phosphate buffer and 75% acetonitrile. The buffer solution was filtered after preparation and the mobile phases were degassed for 5 min before use. The flow-rate was 1.5 ml/min. Fluorescent detector settings were: $\lambda_{ex}$ 345 nm, $\lambda_{em}$ 470 nm, attenuation 2, gain 1000×. The autosampler was equipped with a 99 μl loop. Sample tray temperature was 5°C, and the injection volume was 99 μl for microdialysate and plasma samples, and 45 μl for brain samples. The integrator was set to attenuation 128.

## 3. Results and discussion

### 3.1. Selection of internal standard

Based on pilot investigations FLX was chosen as internal standard because: (1) it is well separated from FEN and NF, (2) its percent recovery from brain and plasma was approximately the same as that of FEN, (3) its fluorescence intensity with Dns-Cl was comparable to that of FEN under the conditions used and (4) no interfering peaks were detectable in blank brain and plasma samples. Vice versa, FEN can be used as internal standard when FLX is the subject of investigation.

### 3.2. Brain microdialysate

Typical chromatograms of microdialysis samples are shown in Fig. 1. Fig. 1A represents a blank microdialysis sample, Fig. 1B represents a microdialysis sample from an animal dosed with 3×5 mg/kg FEN and Fig. 1C is that of microdialysis buffer spiked with NF, FEN and FLX at a concentration of 1 pmol/μl. Standard curves for NF, FEN and FLX spiked together into microdialysis buffer at increasing concentrations were linear between 20 fmol/μl and 2 pmol/μl for FEN and NF. Linearity of FLX was demonstrated between 50 fmol/μl and 2 pmol/μl. The lowest concentrations of these standard curves also represent the minimum quantifiable limits which were 200 fmol (=40.6 and 46.2 pg) for NF and FEN, respectively, and 500 fmol (=154.5 pg) for FLX. The adjusted coefficient of determination for the linear regressions ranged from 0.985 to 0.987. Intra- and inter-assay precision and

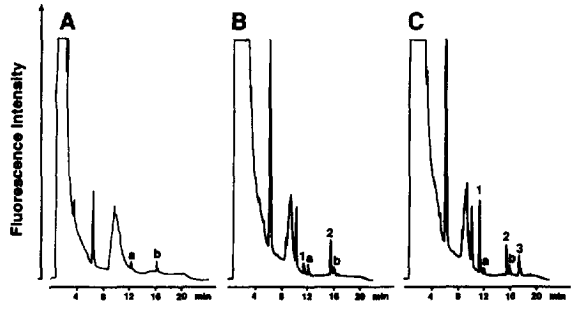


Fig. 1. Typical chromatograms showing the separation of Dns-Cl derivatives in brain microdialysate. Peak No. 1=D-norfenfluramine, No. 2=D-fenfluramine, No. 3=fluoxetine. (A): derivatized blank microdialysate, (B): sample from an animal dosed with 3×5 mg/kg D-fenfluramine, (C): D-norfenfluramine, D-fenfluramine and fluoxetine spiked into microdialysis buffer at a concentration of 1 pmol/μl. 10 μl of microdialysate were reacted with 90 μl of Dns-Cl (1.67 m*M* in acetonitrile). Unknown peaks (labeled "a" and "b") eluted shortly after D-norfenfluramine and D-fenfluramine; however, no interferences occurred.

Table 2
Intra- and inter-assay validation for fenfluramine, norfenfluramine and fluoxetine (R.S.D. of 5 determinations each)

| Concentration | Intra-assay | | | Inter-assay | | |
|---|---|---|---|---|---|---|
| | Microdialysate | Plasma | Brain | Microdialysate | Plasma | Brain |
| *D-Fenfluramine* | | | | | | |
| Low | 8.3% | 1.6% | 8.8% | 12.6% | 11.5% | 10.0% |
| High | 1.4% | 1.3% | 0.4% | 6.6% | 6.9% | 9.3% |
| *D-Norfenfluramine* | | | | | | |
| Low | 0.6% | 1.5% | 0.7% | 3.6% | 11.8% | 21.4% |
| High | 0.5% | 1.6% | 0.3% | 4.6% | 3.7% | 7.9% |
| *Fluoxetine* | | | | | | |
| Low | 3.1% | 1.0% | 4.8% | 12.0% | 14.9% | 25.3% |
| High | 0.5% | 1.2% | 0.6% | 10.4% | 4.9% | 12.2% |

Low=0.2 pmol/μl microdialysate, plasma, 5 pmol/mg brain.
High=2 pmol/μl microdialysate, plasma, 50 pmol/mg brain.

accuracy are listed in Table 2. Intra-assay precision varied from 0.5 to 8.3%, and inter-assay precision varied from 3.6 to 12.6%, depending on concentration and analyte.

Retention times of NF, methamphetamine (for the purpose of comparison), FEN and FLX were 11.45, 12.45, 15.60 and 17.60 min, respectively. Fig. 2 illustrates that the primary amine (NF) gave the highest fluorescent yield as compared to the secondary amines. However, this did not translate into a lower limit of quantitation for NF because of the higher background noise in the closer vicinity of the early eluting spurious peaks.

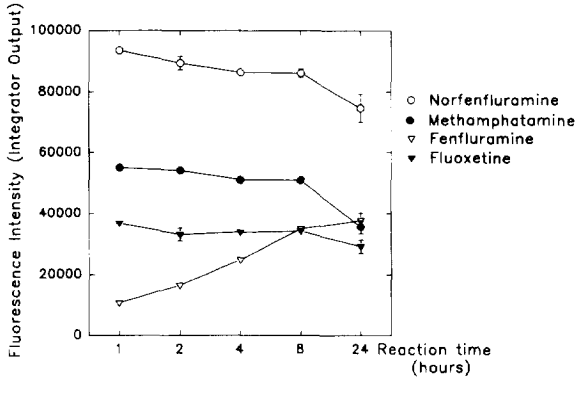


Fig. 2. Dependence of the fluorescent yield of the compound and the length of the derivatization time. 10 μl of standards ($n$=5/time point) containing 20 pmol each of D-norfenfluramine, methamphetamine, D-fenfluramine and fluoxetine were reacted for the time periods indicated.

### 3.3. Plasma

Typical chromatograms of plasma samples are shown in Fig. 3. Fig. 3A represents a blank plasma sample. Fig. 3B represents a sample from an animal dosed with 3×5 mg/kg FEN and Fig. 3C is that of a plasma sample spiked with NF, FEN and FLX at a concentration of 500 fmol/μl. The regressions were linear between 1 and 200 pmol/100 μl for all three analytes with adjusted coefficients of determination between 0.985 and 0.987. The lowest concentrations of these standard curves also represent the minimum quantifiable limits. Intra-assay precision varied from

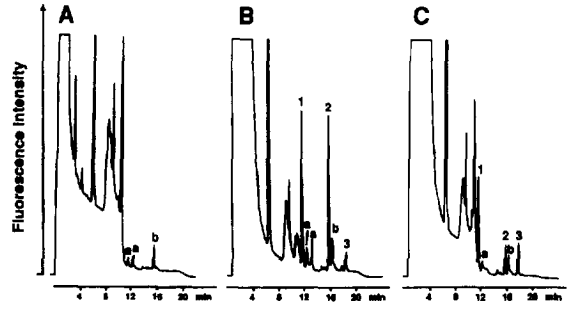


Fig. 3. Chromatograms of Dns-Cl derivatives of extracted plasma samples. (A): Derivatized blank sample, (B): sample from an animal dosed with 3×5 mg/kg D-fenfluramine, fluoxetine added as internal standard, (C): D-norfenfluramine, D-fenfluramine and fluoxetine spiked into plasma so that the concentration in plasma was 50 pmol/100 μl. For further explanations see legend to Fig. 1.

1.0 to 1.6%, and inter-assay precision varied from 3.7 to 14.9% depending on concentration and analyte (Table 2). The percent recovery was comparable for samples of 0.2 and 2 pmol/μl concentration. The recoveries were essentially complete for FEN and NF and somewhat lower for FLX (Table 4). Assuming that the sensitivities of previously reported methods were based on a 1 ml volume of plasma this would translate into absolute minimum quantifiable amounts of 8–9 pmol for FEN [6,10] and 3 pmol for FLX [17]. The present method yielded a sensitivity of 2 pmol for FLX and a sensitivity of 1 pmol for FEN and NF from a starting volume of only 90 μl of plasma.

### 3.4. Brain tissue

Typical chromatograms of brain tissue samples are shown in Fig. 4. Fig. 4A represents a sample of blank brain tissue. Fig. 4B represents a sample from an animal dosed with 3×5 mg/kg FEN and Fig. 4C is that of brain tissue spiked with NF, FEN and FLX at a concentration of 2 pmol/mg (Fig. 4C). All three compounds were well quantifiable at a concentration of 5 pmol/mg tissue. Regressions of extracted brain standards were linear between 5 and 200 pmol/mg with adjusted coefficients of determination between 0.985 and 0.987. The percent recoveries were about

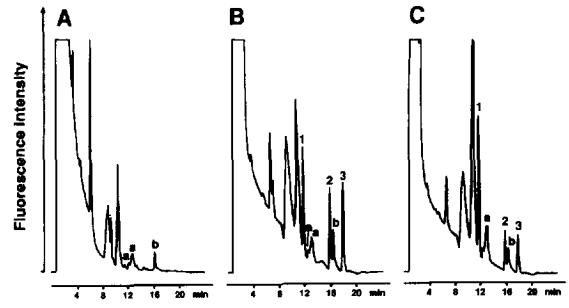


Fig. 4. Chromatograms of Dns-Cl derivatives of extracted brain tissue samples. (A): Derivatized blank sample, (B): sample from an animal dosed with 3×5 mg/kg D-fenfluramine, fluoxetine added as internal standard, (C): D-norfenfluramine, D-fenfluramine and fluoxetine spiked into borate buffer diluted brain homogenate so that the theoretical concentration in brain tissue was 20 pmol/10 mg. For further explanations see legend to Fig. 1.

50% for FEN or FLX and 80% for NF (Table 4) with only slight (additional 5–10%) improvements gained by a second extraction (data not shown).

### 3.5. Stability of derivatives

The stability of dansylated NF, FEN and FLX (2 pmol/μl) after refrigerated storage in the dark is depicted in Table 3. The Dns-Cl derivatives were virtually unchanged over a period of 24 h. These results verify other reports [25,27] concerning the

Table 3
Extraction recovery for plasma and brain samples

| Concentration (μM) | Reference PKHT | Extracted PKHT | Recovery (%) |
|---|---|---|---|
| *D-Fenfluramine* | | | |
| 0.2 (plasma) | 28 563 | 32 316 | 113 |
| 2 (plasma) | 282 978 | 307 004 | 108 |
| 5 (brain) | 427 933 | 206 434 | 48 |
| *D-Norfenfluramine* | | | |
| 0.2 (plasma) | 95 683 | 93 161 | 97 |
| 2 (plasma) | 967 199 | 1 016 576 | 105 |
| 5 (brain) | 1 087 385 | 846 825 | 78 |
| *Fluoxetine* | | | |
| 0.2 (plasma) | 37 622 | 29 785 | 79 |
| 2 (plasma) | 411 932 | 385 180 | 94 |
| 5 (brain) | 485 217 | 218 065 | 45 |

Spiked plasma and brain samples were extracted. After reconstituting the dried extracts with 10 μl of sodium phosphate buffer (pH 2.5), 7–8 mg solid $NaHCO_3$ and 90 μl Dns-Cl solution were added. The reference standard data were produced by reacting 10 μl standard solution (0.2, 2 or 5 pmol/μl) with 90 μl of Dns-Cl solution. Values represent averages of $n=4$ for each concentration. Injection volume was 99 μl for plasma and 49.5 μl for brain samples. Peak height (PKHT) was corrected for the percent solvent volume used during the extraction procedure (see Section 2.5).

Table 4
Stability of Dns-Cl derivatives

| Compound | Percent fluorescence intensity of samples injected immediately | | |
|---|---|---|---|
| | 4 h | 8 h | 24 h |
| FEN | 100±2 | 96±3 | 98±3 |
| NF | 103±2 | 99±2 | 115±3 |
| FLX | 101±2 | 95±2 | 98±4 |

Derivatized standards containing D-fenfluramine (FEN), D-norfenfluramine (NF) and fluoxetine (FLX) were stored at 4°C in the autosampler for the time indicated below after being reacted for 4 h ($n$=4 per time point, 2 pmol/μl, Mean±S.D.).

stability of these products. Dansylated NF, FEN and FLX proved to be clearly more stable than the *o*-phthaldialdehyde/3-mercaptopropionic acid derivatives of D-amphetamine, *p*-hydroxy-D-amphetamine and tryptamine we investigated earlier [22].

### *3.6. Effect of reaction time on fluorescent yield*

Previously it had been reported that the fluorescence yield was maximal when derivatization took place at room temperature [25]. Therefore we focused on room temperature for optimizing the reaction time. Fig. 2 demonstrates that the duration of derivatization (at room temperature) affected the fluorescent yield differently for the individual compounds. Whereas FENs fluorescent yield increased constantly over 24 h, decreases were observed for three other compounds, including NF and FLX. The decrease was most pronounced for NF. These findings are different from those reported by others for dansylated cyclosporin A [24], ammonia and methylamine [25], and taurine [27]. The present findings lead to the conclusion that optimum conditions for Dns-Cl derivatization can differ between analytes. It is known that the metabolite (NF) is often present in lower concentrations in animals and humans as compared to the administered FEN. Therefore, we selected as a compromise a four hour reaction time for the standard application of our method.

In conclusion, the present method is suitable for the analysis of FEN and NF in very small samples (μl and mg range) of microdialysate, plasma and brain tissue with sensitivities comparable to or better than those reported earlier. Although the focus of our investigation was on FEN and NF this method is equally useful for the analysis of FLX. The problem of the excessively large spurious peak(s) supposedly resulting from dansyl chloride hydrolysis [25] which interfered with the detection of NF was resolved by using Macro-Prep 50 Q strong anion-exchange support (resin).

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