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Simple and sensitive high-performance liquid chromatographic determination of methamphetamines in human urine as derivatives of 4-(4,5-diphenyl-1H-imidazol-2-yl) benzoyl chloride, a new fluorescence derivatization reagent

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Abstract

A simple, rapid and highly sensitive high-performance liquid chromatographic method for the determination of methamphetamine and its metabolites in human urine was developed. Without tedious pretreatment procedures, aliquots of methamphetamine spiked or abusers' urine samples were simply acidified, dried under a stream of N_2 gas, and then derivatized with 4-(4,5-diphenyl-1H-imidazol-2-yl)benzoyl chloride in acetonitrile in the presence of traces of triethylamine. Five derivatives were isocratically separated within 33 min by an ODS column, and the effluent was monitored at 440 nm (λ_{ex}, 330 nm). Using 1-phenylethylamine as an internal standard calibration curves were confirmed to be linear up to at least 2×10^{-5} *M* in urine with correlation coefficients of 0.998–1.000, while the detection limits at $S/N=3$ were 0.6–5.2 fmol per 5-μl injection. The relative standard deviations ($n=5$) of inter- and intra-day variations were less than 8.9%. The correlation between the concentrations of methamphetamine and amphetamine in urine determined by the proposed method and another currently accepted one was satisfactory. The method was successfully applied to urine samples collected from methamphetamine addicts.

Keywords: Human urine; Methamphetamine; 4-(4,5-Diphenyl-1H-imidazol-2-yl)benzoyl chloride; Lophine analogs

1. Introduction

Among the controlled sympathomimetic amines that are known to increase the motorial activity, generate feelings of energy, euphoria and heightened mental awareness, methamphetamine (MP) and amphetamine (AP) are the most widely abused compounds in Japan and Sweden, respectively [1,2]. Therefore, developing simple, selective and highly sensitive analytical techniques to quantify these indirectly acting adrenotnimetics in biological samples is still rapidly growing and interesting to many researchers. Thus, various methods to determine these compounds have been developed, and among them, HPLC methods [3–9] after derivatization with a fluorogenic or chemiluminogenic reagent have shown superior sensitivity in comparison with other methods such as HPLC—UV [10,11], gas chromatography (GC) [2,12,13], gas chromatography–mass

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spectrometry (GC–MS) [14–16], or polarization fluoroimmunoassay methods [17,18].

Recently, we synthesized and evaluated the chemiluminescence and fluorescence properties of derivatives of lophine (i.e. 2,4,5-triphenyl imidazole) [19] which has been first reported as a potential chemiluminogenic compound in 1877 by Radziszewski [20]. Among these, 4-(4,5-diphenyl-IH-imidazol-2-yl) benzoyl chloride (DIB-Cl) showed almost equal chemiluminescence intensity compared to the parent compound lophine, and its fluorescence intensity (FI) was ca. 24 times stronger than that of the latter [19].

In the previous reports [3,21], two fluorogenic reagents for amines have been investigated, and we concluded that 4-fluoro-7-nitrobenzofurazan (NBD-F), unlike fluorescein isothiocyanate (FITC), is unsuitable for the simultaneous sensitive determination of primary and secondary sympathomimetic amines, while the sample pretreatment and HPLC conditions required for FITC derivatives incited us to develop a more sensitive and simple method. Consequently, in this study, DIB-Cl, a lophine analogue, which was first synthesized in our laboratory, was investigated as a fluorogenic reagent to derivatize MP, AP, *para*-hydroxymethaphetamine (*p*-HMP), *para*-hydroxyamphetamine (*p*-HAP), ephedrine (EP), hereafter referred as methamphetamines (MPs), and 1-phenylethylatnine (PEA) as an internal standard (I.S.).

2. Experimental

2.1. Materials and reagents

DIB-Cl was synthesized in our laboratory as reported previously [19]. MP·HCl and EP were obtained from Dainippon Pharmacy (Osaka, Japan), and PEA was obtained from Nacalai Tesque (Kyoto, Japan). AP sulphate, *p*-HAP·HCl and *p*-HMP·HCl were synthesized in our laboratory according to known procedures [22,23]. Dansyl-Cl (Dns-Cl) and HPLC-grade acetonitrile were purchased from Wako (Osaka, Japan). NBD-F and a reagent-grade tris–(hydroxymethyl) aminomethane (Tris) were obtained from Dojindo laboratories (Kumamoto, Japan) and Sigma (St. Louis, MO, USA), respectively. Water was deionized and passed through a pure-line WL2lP (Yamato Kagaku, Tokyo, Japan). All other chemicals were of analytical reagent grade and used as received without further purification.

Standard stock solutions of MPs were prepared by dissolving the proper amounts of these compounds in DMF to give a final concentration of 1×10^{-2} *M* per compound. These solutions were kept at 4°C and were stable for at least 3 months. Working solutions were prepared by dilution in acetonitrile for derivatization of standard samples, and in water to spike the urine samples.

Spiked urine samples were prepared by adding aliquots of MPs standard solutions to fresh, drug-free urine collected from healthy subjects in our department. Urine samples from MP addicts were kindly supplied by the Forensic Laboratory of Nagasaki Prefectural Police Headquarters, and were kept at −20°C until needed.

Tris–HCl buffer was prepared by adjusting the pH with concentrated HCl to 7.0, and then adjusting the volume to give a final concentration of 100 m*M*.

2.2. Samples pretreatment and derivatization reaction

Urine samples were spiked with aliquots of 2.5×10^{-3} *M* of the I.S. and diluted 10-fold with water to give a final PEA concentration of 2.5×10^{-6} *M*. A portion of 10 μl of urine was transferred to a screw-capped reaction vial, mixed with 10 μl of acetic acid and then dried under a gentle stream of N_2 gas at room temperature. After reconstituting the residue in 100 μl acetonitrile, 200 μl of 30 μ*M* DIB-Cl in acetonitrile, and 100 μl of 72 μ*M* TEA in acetonitrile were added successively, vortex-mixed for 1 min and placed in dark at room temperature (ca. 24–27°C) for 30 min, then 5 μl of the reaction mixture were injected onto the column. For the standard samples, 100 μl of MPs in acetonitrile were used.

Solid-phase extraction (SPE) following known procedures [9,25] was carried out as follows: to 2 ml of urine or diluted urine containing 1×10^{-6} *M* *p*-HAP, *p*-HMP, EP, AP, MP and PEA, 2 ml of boric acid–NaOH buffer (50 m*M*, pH 8.0) were added. The mixture was applied onto a Sep-pak C_{18} cartridge, washed with 10 ml of water, and eluted with

4 ml of methanol. After adding 20 μl of acetic acid, a 200-μl portion of the eluate was dried up under N_2 gas at room temperature. The residue was reconstituted in 100 μl of acetonitrile and used for the derivatization reaction with DIB-Cl.

2.3. Measurement of fluorescence

An RF-450 spectrofluorophotometer (Shimadzu, Kyoto, Japan) with a 10-mm quartz cell was used to determine the fluorescence properties of DIB derivatives.

Besides DIB-Cl, standard MP and AP solutions were derivatized also with NBD-F and Dns-Cl and then analyzed on our system under their optimal conditions as reported [6,24].

2.4. HPLC system

The HPLC system consisted of an LC10AS HPLC pump (Shimadzu), a 7125 injector with a 5-μl loop (Rheodyne, Cotati, CA, USA), an analytical column (250×4.6 mm I.D., 5 μm: Daisopak SP-120-5-ODS, Daiso, Osaka, Japan), an RF-550 spectrofluorometer (Shimadzu), set at λ_{ex} and λ_{em} of 330 and 440 nm, respectively, and a U-228-2P-500 recorder (Nippon Denshi Kagaku, Tokyo, Japan). A 0.5-mm stainless steel tubing was used in all flow lines.

The HPLC separation was carried out by using a mixture of Tris–HCl buffer (100 m*M*, pH 7.0)–1% isopropanol in acetonitrile (45:55, v/v) as the mobile phase. The eluent was premixed, passed through a 0.2-μm JG type membrane filter (Nihon Millipore, Yonezawa, Japan) and degassed prior to use.

2.5. Calibration curves

Fresh, drug-free human urine containing 2.5×10^{-5} *M* of the I.S., was spiked with MP, AP, EP, *p*-HAP, and *p*-HMP and diluted 10-fold with water to provide final concentrations in the range of 5×10^{-8} to 2×10^{-5} *M* per compound. These samples were analyzed as described above. Calibration curves for each compound were obtained by plotting the relative fluorescence intensity (RFI) defined as the peak-height ratio of the compound to that of the I.S. against the concentration in urine.

2.6. Validation of the method

The inter- and intra-day variations of the proposed method were evaluated using a fresh, drug-free urine containing 2.5×10^{-6} *M* I.S. spiked with MPs at a concentration of 5×10^{-6} *M* per compound. Five or more samples were analyzed as described over a period of 9 days.

The present method was checked against another currently accepted one (i.e. HPLC with fluorescence detection after Dn_S-Cl derivatization). Fresh, drug-free urine samples containing the I.S. were spiked with AP and MP to give concentrations over the range of 1–20 μ*M* as well as a blank. From every spiked sample, 10 μl were taken and processed as described to be derivatized with DIB-Cl. In parallel, 1 ml of the same urine was taken, pretreated, derivatized with Dns-Cl, and injected onto the HPLC as described in the literature [6].

3. Results and discussion

3.1. Fluorescence properties of DIB-Cl

The fluorescence properties of the reaction mixture of DIB-Cl with MPs were studied in methanol–water (73:27, v/v) and Tris–HCI (100 m*M*, pH 7.0)–acetonitrile (55:45, v/v) mixtures, because DIB derivatives were separable by those two mobile phases. The FI in a methanol–water mixture was about 62% of that in an acetonitrile–Tris–HCl one, while optimal λ_{ex} and λ_{em} were 330 and 440 nm, respectively, in both mobile phases. For comparison purposes, we derivatized AP and MP with two other fluorescence reagents for amines (i.e. Dns-Cl and NBD-F) and analyzed them on our system under their optimum reaction, separation and detection conditions exactly as reported in the literature [6,24]. As shown in Table 1, DIB-Cl required not only milder reaction conditions, but also it showed FI higher than the other reagents.

3.2. Derivatization with DIB-Cl

The derivatization reaction (Fig. 1) was optimized using a 1×10^{-6} *M* standard MPs solution. The highest and constant derivatization yield calculated

Table 1
Comparison of the FI and reaction conditions of MP and AP derivatives of 3 different fluorophores analysed on the same HPLC system

Reagent	Amount on column (pmol)		Peak height (arbitrary units) (%)		FI[a] (%)		Reaction conditions	Mobile phase	K'	
	AP	MP	AP	MP	AP	MP			AP	MP
Dns-Cl[b]	1.67	1.67	10	9	9	17	60 min at 45°C	1 m*M* imidazole in CH_3CN–H_2O, 7:3 v/v (pH 7 adjusted with HNO_3)	6.5	10.8
NBD-F[b]	1.67	1.67	31	1	27	2	10 min at 80°C	1 m*M* imidazole in CH_3CN–H_2O, 6:4 v/v (pH 7 adjusted with HNO_3)	6.0	8.0
DIB-Cl	0.83	0.83	58	27	100	100	30 min at room temperature	1% isopropanol in CH_3CN–Tris–HCl buffer (0.1 *M*, pH 7), 55:45 v/v	12.3	14.7

[a] The FI as peak height of DIB derivatives was considered to be 100.
[b] Ref. [6].

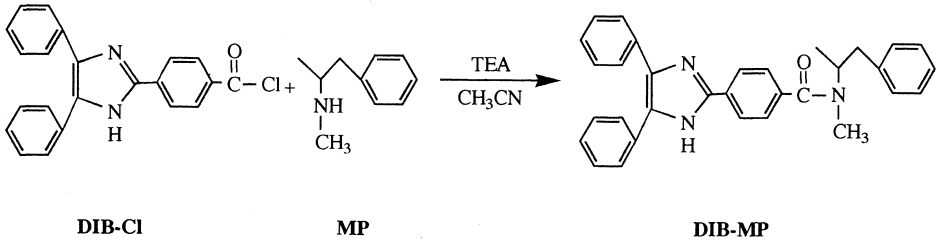


Fig. 1. Reaction scheme for labelling of MP with DIB-Cl.

from the peak heights was obtained at room temperature (ca. 25°C) for 30 min (Fig. 2). At higher temperatures (i.e. 60, 90°C), the derivatization yield of MP, AP, EP and PEA gradually decreased as the temperature increased, while the FI as peak height of *p*-HAP and *p*-HMP became extremely low at 90°C. This might be due to the increase of the reactivity of the phenolic group, another functional group that reacts with DIB-Cl at elevated temperatures, which will end in the formation of dilabeled adducts that fluoresce at different wavelengths and are eluted at different retention times. TEA concentration was examined over the range of 0 to 7.2 m*M* (0 to 0.1%, v/v) in acetonitrile. In samples containing no TEA, the derivatization yields of MP, AP, EP, *p*-HAP, and *p*-HMP were in the range of 6–22% of the highest and constant yields that were observed at a concentration ≥72 μ*M* (0.001%, v/v) or more. Moreover, there was a significant decrease of the interfering peaks when lower TEA concentrations were used. When the concentration of DIB-Cl over the range of 5–100 μ*M* was examined, the highest

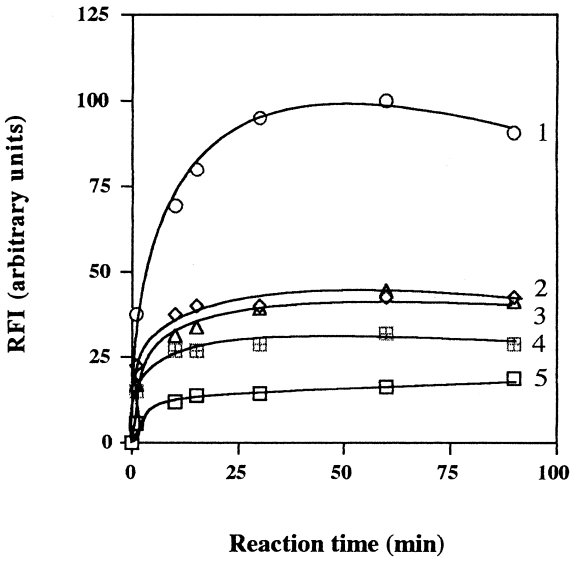
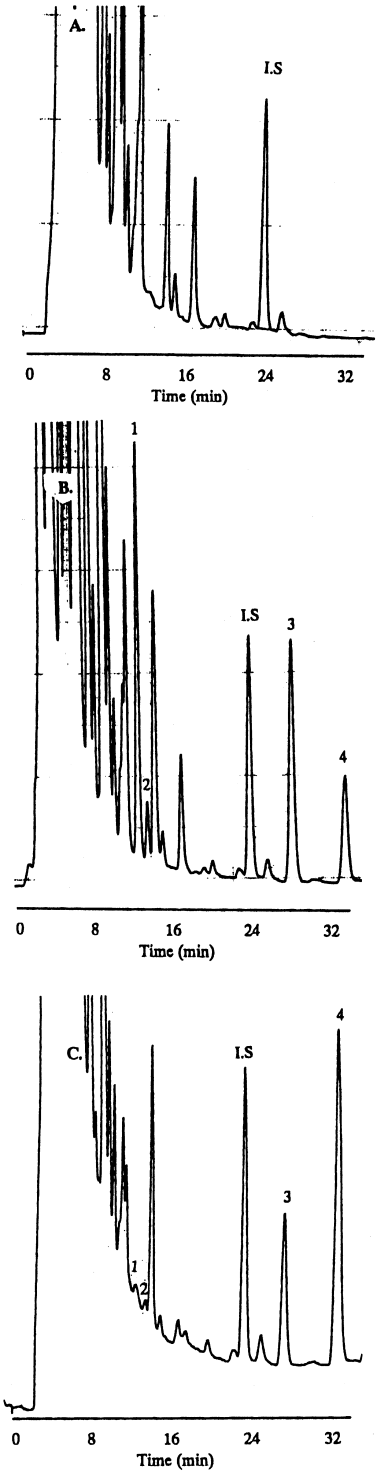


Fig. 2. Reaction time-course of MPs (1 μ*M* per compound) with DIB-Cl (30 μ*M*) at room temperature. For experimental details refer to Section 2.2.



derivatization yield was obtained at a concentration range of 20–30 μ*M* and it decreased at higher concentration. The reason behind that is under investigation, and probably due to the minute amounts of TEA (i.e. 100 μl of 72 μ*M*), that is to say, there might be an optimum molar ratio between TEA and DIB-Cl. DIB-Cl was stable in acetonitrile at least for 1 month when kept in the dark at room temperature, hence, CH_3CN was chosen to be the reagent's solvent. Accordingly, the reaction conditions were chosen as follows; time and temperature, 30 min at room temperature in dark, 100-μl sample, 200 μl of 30 μ*M* DIB-Cl, 100 μl of 72 μ*M* TEA with acetonitrile as solvent for all. The derivatives were confirmed to be stable for at least 24 h when kept in the dark at room temperature.

3.3. Pretreatment of urine samples

From the literature [3,5], the concentrations of MP found in addicts' urine samples were in the range of 9.15×10^{-7} to 1.34×10^{-3} *M*. Therefore, in a sensitive system, the volume of urine expected to be used is very small. In this experiment, we successfully attempted to derivatize MPs contained in spiked and abusers urine samples by simple dilution with water and drying under N_2 gas at room temperature after acidification to avoid evaporative losses. Because the final volume of urine used was in the range of 0.1–3.3 μl, the resultant chromatograms and derivatization yields were almost identical to those of standard solutions. However, following known SPE procedures [9,25], the recovery rates of MP, AP, EP, *p*-HAP, and *p*-HMP from urine at the concentration of 1×10^{-6} *M* per compound ranged from 71–95%.

3.4. HPLC separation

DIB derivatives of MPs were isocratically separable on a reversed-phase C_{18} column within 33 min.

Fig. 3. Typical chromatograms with fluorescence detection of (A) control human urine containing 2.5×10^{-6} *M* I.S.; (B) human urine containing I.S. spiked with MPs at a concentration of 5×10^{-6} *M* per compound; peaks, 1, *p*-HAP, 2, EP, 3, AP and 4, MP; with 625 fmol of each compound injected on the column; (C) MP abuser urine sample number 5. For other experimental conditions refer to Sections 2.2 and 2.4.

Fig. 3 shows typical chromatograms of human urine control, urine spiked with MPs at a concentration of 5×10^{-6} *M* per compound, and a urine sample collected from a MP abuser. Peaks corresponding to DIB-*p*-HAP, -EP, -PEA, -AP and -MP were well separated and eluted at 12, 13.2, 22.8, 27.2 and 32.4 min respectively, in both spiked and MP addict urine. The peak corresponding to DIB-*p*-HMP co-eluted with another unknown peak associated with the reagent blank. DIB-*p*-HMP might be separated if a gradient elution or more polar mobile phase were included in the system, but our purpose to develop a simple and non time-consuming method, made us to sacrifice the quantitation of *p*-HMP.

3.5. Calibration curves and detection limits

Calibration curves were prepared by plotting the peak-height ratio of the target compound to the I.S. against the concentration in urine. Linear relationships were obtained in the concentration range of 5×10^{-8}–2×10^{-5} *M* for *p*-HAP and 1.25×10^{-7}–2×10^{-5} *M* for AP, MP and EP. The regression equations and correlation coefficients (*r*) for each compound were as follows: *p*-HAP, $y=0.325x-0.035$ ($r=1.000$), EP, $y=0.039x-0.001$ ($r=0.998$), AP, $y=0.227x-0.025$ ($r=0.999$), MP, $y=0.093x+0.002$ ($r=0.999$), with *y* as the RFI in arbitrary units and *x* as the concentration in urine in μ*M*. The lower detection limits ($S/N=3$) were 5.0×10^{-9}, 4.2×10^{-8}, 2.1×10^{-8} and 3.1×10^{-8} *M* in urine which are equivalent to 0.6, 5.2, 2.6 and 3.9 fmol on column for *p*-HAP, EP, AP and MP, respectively.

3.6. Validation of the method

The intra- and inter-day variations were assessed using urine samples spiked to give a concentration of

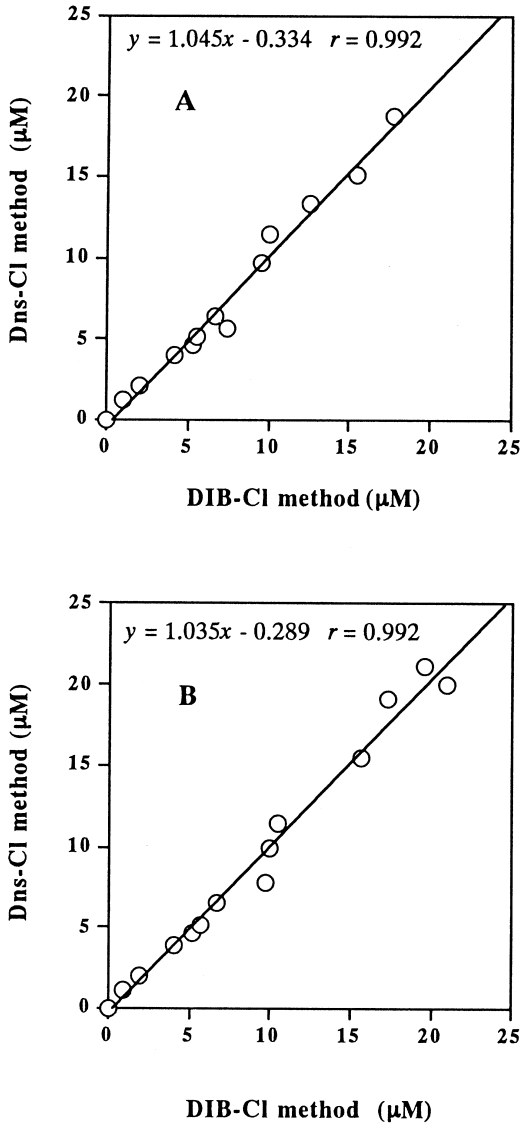


Fig. 4. Correlation between the concentrations of (A) MP ($n=13$) and (B) AP ($n=14$) in human urine determined by the DIB-Cl and Dns-Cl methods.

Table 2
Intra- and inter-day reproducibility of the analysis of MPs in human urine

Compound	Concentration added (μ*M*)	Intra-day ($n=5$)		Inter-day ($n=5$)	
		Found (μ*M*)	% R.S.D.	Found (μ*M*)	% R.S.D.
p-HAP	5.0	5.0	2.5	5.2	3.6
EP	5.0	4.7	4.4	5.3	8.9
AP	5.0	4.5	3.2	4.6	2.3
MP	5.0	4.7	4.4	5.0	3.0

Table 3
Concentrations of MPs determined in urine samples from abusers

Sample	Age	Gender	Concentration in urine (μM)			
			p-HAP[a]	EP[a]	AP[b]	MP[b]
1	44	M	33.7	427	73 (3.4)	1433 (8)
2	31	F	3.2	43.8	9 (8.4)	189 (9.8)
3	37	M	3.6	27.5	21.7 (7.8)	103 (6.6)
4	32	M	2.6	7.5	2.9 (2.7)	3.2 (8.5)
5	32	M	12.3	79.5	189 (16)	1047 (13)
6	44	F	9.4	ND[c]	14 (1.8)	51 (6.2)

[a] Average of duplicates.
[b] Average of triplicates with values in parentheses indicating the % R.S.D..
[c] Not detectable.

5×10^{-6} *M* per compound. Five or more samples were processed as described over a period of 9 days. Table 2 shows the means and relative standard deviations (R.S.D.) of MPs concentrations determined in these samples.

As shown in Fig. 4, the concentrations of MP ($n=13$) and AP ($n=14$) determined in spiked urine samples by the proposed method were checked against Dns-Cl method. Regression lines with satisfactory slopes of 1.035 and 1.045 as well as intercepts of 0.289 and 0.334 for AP and MP, respectively, were obtained, with $r=0.992$ for both compounds.

3.7. Determination of MPs in addict urine samples

The practicality of the proposed method was evaluated using 6 urine samples collected from MP abusers. Table 3 shows the quantitative results of MP and its major metabolite AP, along with *p*-HAP in abusers' urine. EP was also detected in some samples but it is believed to originate from cold medications rather than being a MP metabolite [5]. The high concentrations of MPs usually found in addicts' urine enabled us to use a minimum volume of urine (i.e. 0.1 μl for samples 1 and 5, while 0.67, 0.4, 1.0 and 3.3 μl were used for samples 2, 3, 4 and 6 respectively).

4. Conclusion

In its first application as a fluorogenic reagent, DIB-Cl has been proved to be superior to some other commercially available labelling reagents for amines. This fact is supported by its selectivity, high fluorescence quantum yield, reactivity with primary and secondary amines at room temperature within a reasonable period of time, and what is more important, is its excellent stability in acetonitrile at room temperature for at least 1 month. The sensitivity, sufficient linearity, satisfactory intra- and inter-day precision, applicability to urine samples collected from addicts with very simple pretreatment, good correlation with a currently accepted method and the nontime-consuming isocratic HPLC system reflect the useful potentials of the proposed method in forensic, pharmacokinetics and toxicology studies. Moreover, the reactivity of DIB-Cl with another group of compounds (i.e. phenols) in biosamples is under examination in our laboratory, and the results will be published elsewhere in due course.

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