Automated pre-column derivatization of amines in biological samples with dansyl chloride and with or without post-column chemiluminescence formation by using TCPO–H_2O_2

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On-line automation of two different liquid chromatographic procedures, a pre-column derivatization system and a pre- and post-column system, in order to generate chemiluminescence is reported. Dansyl chloride (Dns-Cl) was used as a pre-column reagent to form fluorophores and bis(2,4,6-trichlorophenyl) oxalate (TCPO) and hydrogen peroxide (H_2O_2) as a post-column reagent to generate chemiluminescence. This procedure is based on the employment of a primary column packed with C_{18} material inserted in a multi-dimensional assembly for sample clean-up and derivatization with Dns-Cl. The dansyl derivatives formed are transferred and separated in a LiChrospher 100 RP_{18} analytical column (125 × 4 mm id, 5 μm film thickness) using acetonitrile–imidazole buffer (pH 6.8) (70 + 30) as eluent. The separated derivatives were transferred to the detector for fluorescence detection or to the post-column system where the chemiluminescence response was generated by using TCPO–H_2O_2 and the products were detected by chemiluminescence. The procedure was optimised for amphetamine and related compounds. A comparison between the on-line pre-column and pre- and post-column systems was performed. The results show that the sensitivity of chemiluminescence detection can be higher than that of fluorescence detection. The recoveries obtained ranged from 98 ± 8 up to 108 ± 8% for amphetamine and methamphetamine, respectively. The accuracy and precision of these methods were evaluated.

Introduction

Chromatography is the most common profiling method for the determination of drugs in biological samples. Gas chromatographic (GC) methods have excellent sensitivity and selectivity, but they often required sample preparation procedures since neither urine nor plasma samples can be introduced directly into a GC system. Several steps are involved in sample preparation for GC, such as deproteinization, extraction of the analytes with organic solvents, sample transfer and preconcentration. These procedures are still performed off-line. The GC-MS method is very sensitive and able to detect femtomole levels, but it needs special and expensive instrumentation.[1,2]

High performance liquid chromatography (HPLC) is commonly used for the measurement of substances in biological materials and the detection of analytes containing amine functional group is routinely carried out with detection limits in the nanogram range by using a fluorescence reagent such as *o*-phthaldialdehyde (OPA),[3] fluorescamine,[4] dansyl chloride (Dns-Cl)[5–8] or 9-fluorenylmethyl chloroformate (FMOC).[9]

Peroxyoxalate chemiluminescence (CL) has been shown to be a highly sensitive detection principle for HPLC, yielding detection limits in the low femtomole and even attomole range.[10–11] Therefore, CL may be regarded as a serious competitor to fluorescence detection in HPLC, GC or GC-MS. Many procedures have been described in which chemiluminescence detection is used for determination of fluorescent derivatives. Reagents such as Dns-Cl,[5–6] naphthalene-2,3-dialdehyde (NDA), anthracene-2,3-dialdehyde (ADA)[12] or luminarin[13] and as a chemiluminescence reagent bis(2,4,6-trichlorophenyl) oxalate (TCPO) and H_2O_2 have been used. In all of these procedures the most tedious and time consuming steps are the sample treatment and fluorescence labelling (pre-column derivatization).

Table 1 summarized the experimental conditions for different pre-column reagents used as fluorophores in the generation of chemiluminescence with TCPO and H_2O_2 *via* a standard dioxethane mechanism for HPLC with chemiluminescence detection and applied to urine samples (except luminarin 1). For instance, Hayakawa *et al*.[6] developed an HPLC method with

Table 1 Comparison between different chemiluminescence procedures and reagents for derivatization of amines in urine samples using peroxyioxalate (TCPO–H_2O_2) as derivatization reagent and HPLC separation

Derivatization reagent (pre-column)	Procedure	Analytes (amines)[a]	Extraction[b]	Reaction time, temperature	Analytical time	Medium	Ref.
Dns-Cl	off-line	1^{er} and 2^{er}	L/L	1 h, 45 °C	2 h	pH 9	6
Dns-Cl	on-line	1^{er} and 2^{er}	—	10 min	22 min	pH 9	This work
NDA	off-line	1^{er}	L/L	2 h, room temp.	3 h	pH 9	6
NBD-F	off-line	1^{er} and 2^{er}	L/L	1 h, 60 ° C	2.5 h	pH 8	6
ADA/NDA	off-line	1^{er}	L/L	20 min, room temp.	1.5 h	pH 9.5	12
Luminarin 1[c]	off-line	1^{er} and 2^{er}	L/L	3 h, 70 °C	4 h	Strictly anhydrous	13

[a] 1^{er} and 2^{er}, primary and secondary amines, respectively. [b] L/L: liquid–liquid extraction of analytes or derivatized analytes. [c] Standard solution.

chemiluminescence detection for amphetamines using Dns-Cl. In this method, before injection into the HPLC system, the amines were extracted from the sample into an organic solvent and derivatized for 1 h at 45 °C, increasing the analytical time considerably. In the procedure using luminarin 1,[13] the reaction time is 20 min at 50 °C and the derivatization reaction has to be performed in a strictly anhydrous medium. The procedure with NDA and ADA[12] required 20 min at room temperature but has the serious disadvantage of the formation of cyanide-induced side-products which are major interferences in reversed phase chromatography and a normal stationary phase has to be used. As can be seen in Table 1, on-line procedures have not been developed and all reagents require long derivatization times and sample extraction (clean-up of the sample or extraction of the derivatives). It is difficult to develop an on-line procedure.

Based on our previous research, in which sample clean-up and amine derivatization were performed off- or on-line using C_{18} solid-phase supports,[14–18] we have recently reported an off-line procedure using dansyl derivatization.[19]

The aim of this study was to study an on-line pre-column derivatization system using fluorescence detection in order to adapt it to an on-line post-column system. The intention was to develop a fully automated procedure with CL detection for the determination of amines in biological samples which achieves very low limits of detection without handling the sample.

Amphetamine and related compounds (amphetamine, methamphetamine and β-phenylethylamine) were chosen as a model compounds. The procedure was applied to the determination of amphetamine and methamphetamine in spiked urine samples as an example of application to real samples.

Experimental

Apparatus

The chromatographic system used consisted of two quaternary pumps equipped with an automatic injector (1050 Series) (Hewlett-Packard, Palo Alto, CA, USA) with a sample loop injector of 100 μl and a high-pressure six-port valve [Rheodyne (Cotati, CA, USA) Model 7000] and column oven. A fluorescence detector (Hewlett-Packard, 1050 Series) (flow cell, 5 μl) was coupled in series and linked to a data system (Hewlett-Packard HPLC ChemStation) that was used for data acquisition and storage. The fluorimeter was used in the chemiluminescence or fluorescence modes. The post-column derivatization instrument (PCX 5100), from Pickering Laboratories (Mountain View, CA, USA), consisted of two isocratic pumps which flushed with variable flow rates. The chromatographic signal was monitored for fluorescence with λ_{exc} = 247 nm and λ_{em} = 510 nm. For the chemiluminescence mode the detector was the same as for the fluorescence mode but the lamp was kept off.

The pre-column and the analytical column were combined by means of a switching arrangement. All the assays were carried out at ambient temperature.

Reagents

All the reagents were of analytical-reagent grade except where indicated otherwise. Acetonitrile, methanol and acetone (Scharlau, Barcelona, Spain) were of HPLC grade. Methamphetamine hydrochloride, β-phenylethylamine hydrochloride, amphetamine sulfate and dansyl chloride were obtained from Sigma (St. Louis, MO, USA), sodium hydrogencarbonate from Probus (Badalona, Spain), sodium hydroxide from Panreac (Barcelona, Spain), TCPO from ICN Biomedicals (Irvine, CA, USA), imidazole (99%) from Sigma and H_2O_2 (30%) reagent grade from Panreac.

Columns and mobile phases

For the dansylation derivatization procedure, a pre-column (20 × 2.1 mm id) dry-packed with Hypersil ODS-C_{18} (30 μm) as stationary phase was used. Purified water was used for washing the pre-column during the clean-up step (mobile phase of pump 1 in Fig. 1) and acetonitrile–imidazole (0.06 M, pH 6.8) (70 + 30 v/v) as the mobile phase of pump 2 in Fig. 1. Acetonitrile (100%) for 2 min followed by water in the gradient mode (up to 100% water) for 2 min (pump 1) was used to clean the pre-column. All the solvents were filtered with a 0.45 μm nylon membrane (Teknokroma, Barcelona, Spain) and degassed with

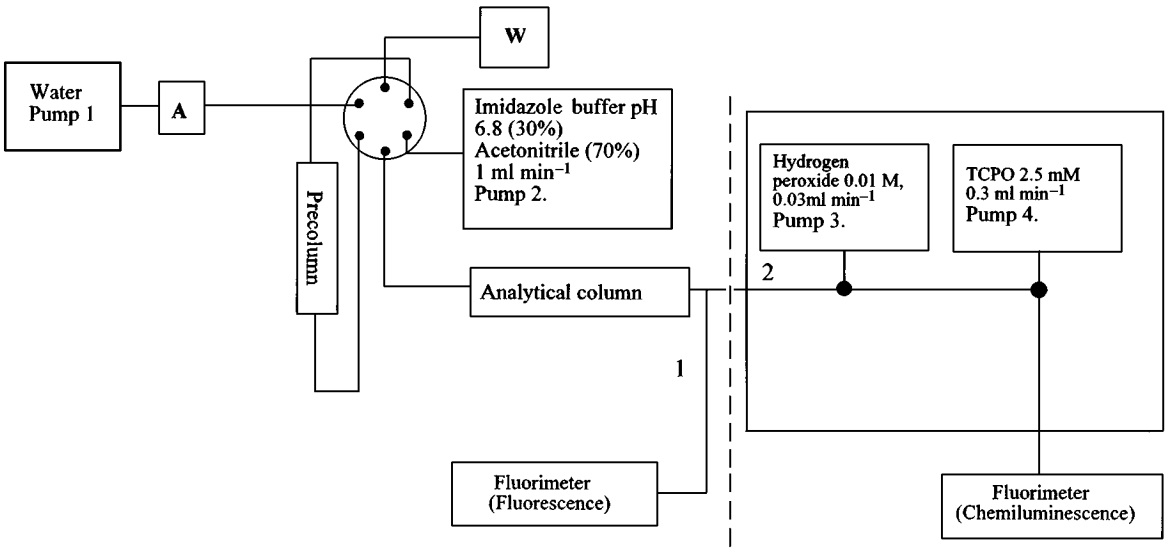

Fig. 1 Scheme of pre- and post-column derivatization of amines by using Dns-Cl and TCPO–H_2O_2 chemiluminescence detection. System 1: on-line dansylation into C_{18} cartridges and fluorescence detection. System 2: on-line pre-column derivatization and post-column chemiluminescence generation with CL detection.

helium before use. For the post-column system, H_2O_2 (10 mM) and TCPO (2.5 mM) were used as the mobile phase of pumps 3 and 4, respectively, at a flow rate of 0.3 ml min^{-1} (Fig. 1).

Preparation of solutions

Standard solutions of Dns-Cl were prepared by dissolving the pure compound in acetone. Stock standard solutions of the amine compounds and amine working standard solutions were obtained by dissolving the pure compounds in water (1000 μg ml^{-1}) and diluting further with water as required. Hydrogencarbonate–carbonate buffer was prepared by dissolving the appropriate amount of sodium hydrogencarbonate in water and then adjusting the pH with 10% m/v NaOH. All solutions were stored in the dark at 2 °C. The reagent solutions of TCPO and H_2O_2 in acetonitrile were prepared daily. Imidazole buffer (0.06 M) was prepared by dissolving the appropriate amount in water and adjusting the pH to 6.8 with nitric acid.

Pre-column derivatization: dansylation

The set-up used for the on-line derivatization of the samples is shown in Fig. 1. At the beginning of each assay, 50 μl of sample were injected on to the trapping column, the switching valve being in position A. Matrix constituents were flushed out by flushing the pre-column with water (delivered by pump 1). After sample injection, the autosampler withdrew 30 μl of Dns-Cl (5 mM) and 70 μl of $NaHCO_3$ (1%, pH 9) buffer from separate vials. The diluted Cl-Dns was injected on to the pre-column. Cleaning of the samples was carried out during the injection of the reagent and buffer. At 2.5 min the flow in pump 1 was zero. After the reaction time (13 min), the switching valve from pump 2 was rotated to position B, and the derivatives were transferred to the analytical column by using the eluent from pump 2. Different volumes of water for flushing the pre-column (V_f) were evaluated. Since the injection programme was the same in all instances, on changing the flow rate in pump 1 V_f is modified, changing the flow rate in pump 1.

At 17 min, the valve was turned to the original position to regenerate and re-equilibrate the pre-column, while the derivative separation into the analytical column was performed. Rotation of the valve was performed automatically.

Derivatizations were carried out at room temperature and each sample was assayed in triplicate.

The separated compounds were transferred to the detector for fluorescence detection or to the post-column system and finally detected by chemiluminescence.

Recoveries studies

Retention of the amphetamines in the trapping column and the percentage of drug recovered after clean-up and derivatization were evaluated by comparing the peak areas obtained with those obtained for direct injection of standards containing an equivalent amount of drug, after off-line solution derivatization.[19] In all cases, each sample was assayed in triplicate.

Post-column generation of chemiluminescence

Pumps 3 and 4 (Fig. 1) were running continuously during all runs at a flow rate of 0.3 ml min^{-1}, with H_2O_2 and TCPO, respectively. The dansyl derivatives, previously separated in the analytical column, were transferred to the post-column system, where the intermediate generated from the TCPO and H_2O_2 served to excite the Dns-amines so that they could then undergo chemiluminescence emission.

Amphetamine and methamphetamine determination in spiked urine samples

Untreated urine samples were spiked with amphetamine, methamphetamine and β-phenylethylamine in the range 0.5–5 μg ml^{-1}. Volumes of 1 ml of these samples and 10 μl of $NaHCO_3$ (pH 10.5) were placed in glass injection vials and 50 μl were injected. The derivatization or/and chemiluminescence formation was performed as described above.

Results and discussion

Fig. 1 shows the derivatization system used, a fully automated procedure with on-line derivatization into a C_{18} packing in the primary column to form dansylated derivatives with the possibility of performing on-line post-column TCPO–H_2O_2 and chemiluminescence detection. This system was used with fluorescence or chemiluminescence detection in which the post-column instrument was included.

Derivatization conditions

Pre-column derivatization (dansylation). The time for reaction between Dns-Cl and both primary and secondary amino groups is fairly long and the complete reaction takes from 10 min to 1 h at basic pH and at high temperatures (70–45 °C).[5–8] We have studied an off-line dansylation derivatization procedure using a C_{18} packing support.[19] This procedure was applied to urine samples and the experimental conditions were room temperature, 5 mM Dns-Cl, acetone–hydrogencarbonate solution (20 mM, pH 9–9.5) (2 + 3 v/v) and reaction time 30 min. Based on the results for the off-line procedure, the conditions for an on-line procedure were studied. Plots of the analytical signal (fluorescence) *versus* reaction time (5–25 min) are shown in Fig. 2(A). As can be seen, on increasing the reaction time the signal increases for the three compounds. However, if a reaction time of 10 min is used, the sensitivity observed is satisfactory for most applications concerning the determination of these compounds.[20]

Increasing the amount of reagent injected can also increase the response [Fig. 2 (B)]. However, there are two possible problems in increasing the excess of Dns-Cl. First, the response of undesirable peaks increase as the amount of reagent is increased. The reagent peaks appear at the beginning of the chromatogram, up to 2 min (see Fig. 3). These interferences could be eliminated by including a clean-up step just after the derivatization and before transferring the reaction products to the analytical column. However, according to our previous paper,[19] these peaks do not interfere directly in the fluorescence determination, so no reagent clean-up step to remove the reagent excess was necessary.

Second, Dns-Cl exhibits low solubility in water and a less polar solvent, such as acetone, for dissolution. Partial elution of the compounds of interest is produced when a large volume of acetone is injected. Dns-Cl was dissolved in acetone–carbonate buffer (20 mM, pH 9) (2 + 3 v/v). As can be seen in Fig. 2(B), the response increased with increase in the volume of reagent injected up to 30 μl; larger volumes did not increase the analytical signal, but the background noise increased. A 30 μl volume of reagent solution (5 mM) was selected for subsequent work. We observed a linear response for aqueous solutions of the analytes in the tested concentration range (0.5–5 μg ml^{-1}). The responses obtained for each analyte were also independent

of the presence of other amines, which means that different compounds can be processed simultaneously.

Carbonate buffer of pH 9 was chosen. The influence of the volume of buffer on the responses was studied in the range 5–90 μl. On increasing the volume injected, the analytical responses increased, but when carbonate buffer was selected instead of water as the mobile phase, the analytical responses of the analytes decreased and the peaks corresponding to side products increased, which suggests than Dns-Cl is rapidly degraded in the presence of the buffer. A 70 μl volume of buffer yielded satisfactory derivatization rates.

The volume of water (V_f) for flushing the pre-column was studied and 2.5 min was selected as optimum (gradient flow from 1 ml to zero). Longer times (>3 min) reduced the analytical responses, probably owing to elution of the analyte.

The effect of the temperature of the reaction was also tested in the range 20–45 °C; the responses increased with increase in temperature. As performing the procedure at room temperature was sensitive enough to determine these analytes, higher temperatures were not needed.

The optimum dansylation conditions were a 50 μl amine sample, 30 μl of Dns-Cl (5 mM), 70 μl of buffer (20 mM $NaHCO_3$, pH 9), 2.5 min (flushing the pre-column, water from 1 to 0 ml) and reaction time 10.5 min. This on-line pre-column derivatization was compared with solution derivatization off-line and recoveries of 84 ± 6 and 50 ± 6% for amphetamine and methamphetamine, respectively, were obtained.

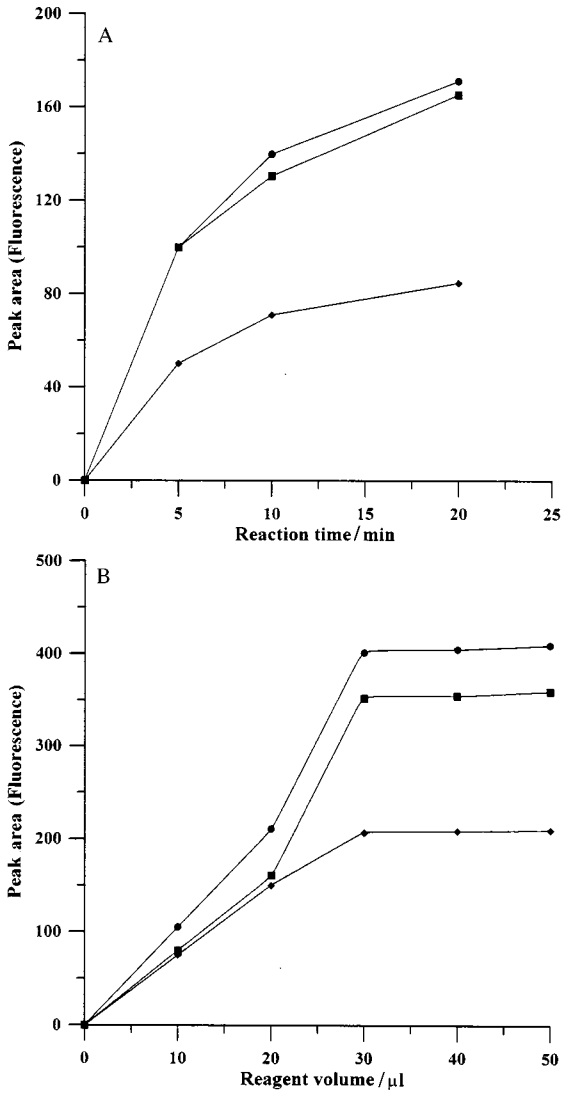

Fig. 2 (A) Effect of time. Conditions: room temperature, 10 μl of reagent, 90 μl of buffer, 50 μl of sample. (B) Effect of the reagent concentration in the pre-column derivatization on peak areas. Conditions: room temperature, 70 μl of buffer, 50 μl of sample, reaction time 10 min. Concentration of analytes: 3.2 μg ml^{-1} (in water) (●) of β-phenylethylamine, (■) amphetamine and (◆) methamphetamine. For experimental details, see Experimental section.

Post-column generation of chemiluminescence. According to our previous work,[19] the conditions for the post-column generation of chemiluminescence were TCPO (2.5 mM) and H_2O_2 (10 mM) dissolved in acetonitrile, imidazole buffer (pH 6.8, 0.06 M), room temperature and flow rates of 0.3 ml min^{-1} of each solvent. After separation into the analytical column, the dansylation derivatives were transferred to the post-column system and finally detected by chemiluminescence (Fig. 1).

The final recommended procedure for derivatization (dansylation) and chemiluminescence generation for amphetamine and related compounds is summarized in Table 2.

Amphetamine and methamphetamine determination in urine with pre-column derivatization with or without post-column chemiluminescence generation on-line

Both procedures, pre-column derivatization with fluorescence detection (A) and pre-column derivatization with post-column chemiluminescence generation with CL detection (B), were applied to the determination of amphetamines (amphetamine and methamphetamine) in spiked urine samples. Fig. 3 shows the chromatograms of a spiked urine sample obtained by applying procedure A. As can be seen, the selectivity is satisfactory, since endogenous urinary compounds are eluted at retention times shorter than those of the analytes.

Table 3 gives the recoveries for the on-line procedures, which were calculated by comparing the results for urine sample with the calibration graph for standard solutions. In both cases the recoveries were nearly 100%, which means that no differences between standard solutions and urine samples were observed. The precision of both procedures was satisfactory.

Table 4 gives the accuracy of the two procedures when spiked urine samples were analysed. As can be seen, the accuracy of the two procedures is good. These analytes can be determined with satisfactory linearity in the range 0.5–10 μg ml^{-1}, thus covering the therapeutic range.[20] The sensitivity and selectivity

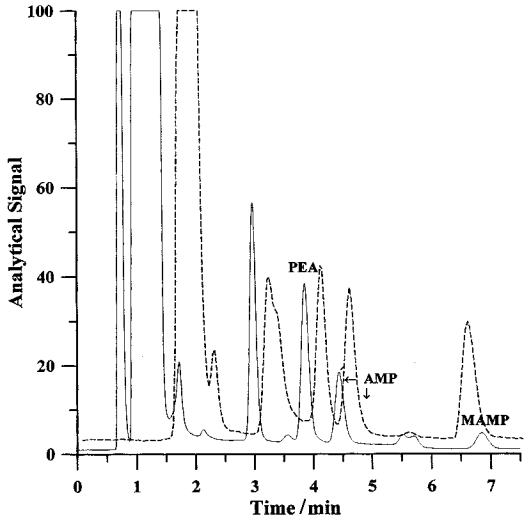

Fig. 3 Chromatograms corresponding to on-line pre-column derivatization with fluorescence detection (solid line) of urine samples spiked with β-phenylethylamine 3.37 μg ml^{-1}, amphetamine 2.83 μg ml^{-1}, methamphetamine 3.49 μg ml^{-1} or with chemiluminescence detection (broken line) (amine concentrations: β-phenylethylamine 2.28 μg ml^{-1}, amphetamine 4.00 μg ml^{-1} and methamphetamine 5.02 μg ml^{-1}). Internal standard: β-phenylethylamine. For experimental details, see Experimental section.

obtained in both procedures are similar for amphetamine. Methamphetamine is more sensitivity determined when chemiluminescence detection is used.

In contrast, methods using off-line solution derivatization gave limits of detection in the low ng ml^{-1} range.[20] However, these methods usually involved other steps in the sample treatment, such as liquid–liquid extraction and preconcentration. On-line procedures seem to be less sensitive, and the limits of detection are in the 0.02–1.0 μg ml^{-1} range.[9,21]

Our procedure using off-line dansylation with a C_{18} packing support with and without chemiluminescence detection has detection limits 1 and 10 ng ml^{-1}, respectively. In this assay (on-line procedure), the detection limits (for a signal-to-noise ratio of three) are also in the range of 1–10 ng ml^{-1} for chemiluminescence and fluorescence detection, respectively, and these can be considered acceptable. Better sensitivity could be achieved by increasing the temperature or reaction time in the pre-column derivatization procedure, or by using larger flow

Table 2 Time schedule and conditions used in the determination of amphetamine and related compounds in urine

Valve position	Cumulative time/min	Trapping column	Analytical column
A	0	Injection of sample: 50 μl	70 + 30 v/v acetonitrile–imidazole buffer
	0–2.5	Injection of Dns-Cl solution, 30 μl Injection of buffer, 70 μl Sample clean-up (gradient from 1 ml to zero)	(flow rate 0.1 ml min^{-1})
	9–13		70 + 30 v/v (acetonitrile–imidazol buffer) (gradient flow rate 0.1–1 ml min^{-1})
B	13–17	Analytical separation 70 + 30 v/v acetonitrile–imidazole buffer	70 + 30 v/v acetonitrile–imidazole buffer (flow rate 1.0 ml min^{-1})
A	17–19	Water–acetonitrile (gradient to 100% acetonitrile)	70 + 30 v/v acetonitrile–imidazole buffer
	20–21	Water–acetonitrile (gradient to 100% water)	(flow rate 1.0 ml min^{-1})
	22	End	70 + 30 v/v acetonitrile–imidazole buffer (gradient flow rate 1–0.1 ml min^{-1})

Table 3 Analytical data for the determination of amphetamine and related compounds in urine samples. For experimental details, see Experimental section. Recoveries determined at half concentration in the tested range

Procedure (derivatization)	Analyte[a]	Recovery (%)[b] (n = 8)	Linearity for urine samples (n = 15)	Inter-day precision (%) (n = 3)	Intra-day precision (%) (n = 8)
Pre-column	AMP	97 ± 4	a = 0.016 b = 0.7892 r^2 = 0.998	3	4
	MAMP	90 ± 3	a = −0.0106 b = 0.40260 r^2 = 0.9990	2	3
Pre- and post-column CL	AMP	98 ± 8	a = 0.016 b = 0.7880 r^2 = 0.998	7	8
	MAMP	108 ± 8	a = −0.0671 b = 0.5660 r^2 = 0.998	5	8

[a] AMP = amphetamine; MAMP = methamphetamine (β-phenylethylamine was used as internal standard). [b] Recoveries based on the calibration graph with standards.

Table 4 Accuracy in the determination of amphetamine and methamphetamine in urine samples by using the two proposed procedures (on-line pre-column derivatization or pre-column derivatization and post-column chemiluminescence generation)

Procedure (derivatization)	Sample	Analyte	Added conc./ μg ml^{-1}	Found conc./ μg ml^{-1} (n = 3)	Error (%)
Pre-column	1	AMP	—	—	
		MAMP	1.13	1.02 ± 0.04	−9.7
	2	AMP	0.70	0.70 ± 0.03	0.0
		MAMP	2.26	2.16 ± 0.47	−4.4
	3	AMP	1.49	1.40 ± 0.06	−6.0
		MAMP	4.49	4.46 ± 0.01	−0.6
Pre- and post-column CL	1	AMP	0.7	0.67 ± 0.03	+4.2
		MAMP	0.91	0.82 ± 0.05	−8.9
	2	AMP	1.45	1.50 ± 0.03	−3.0
		MAMP	1.81	1.92 ± 0.47	+6.1
	3	AMP	2.89	3.09 ± 0.06	−7.0
		MAMP	3.63	3.45 ± 0.01	+5.0
	4	AMP	4.34	4.61 ± 0.02	+5.8
		MAMP	5.44	5.47 ± 0.06	−0.64
	5	AMP	5.8	6.25 ± 0.03	−7.8
		MAMP	7.25	7.68 ± 0.13	+5.9

cells than that used in this work (5 μl) in the fluorimeter. Other on-line procedures have been studied in our research group using reagents such as naphthoquinone 4-sulfonic acid (NQS), OPA and FMOC.[17] The dansylation method has the advantage of requiring less time to clean the column before another injection and a few millilitres of acetonitrile are sufficient to reconstitute the column, and has the possibility of forming chemiluminescence derivatives, which can increase the sensitivity and selectivity.

This work shows for the first time the possibility of realizing on-line dansylation followed by on-line post-column chemiluminescence generation. This option allows direct injection of the urine sample into the chromatographic system; the samples are cleaned and derivatized (pre-column) with Dns-Cl into a C_{18} column in the system and subsequently transferred to the post-column derivatization section (Fig. 1). Hence these are automated procedures that avoid the handling of the sample and improve the analysis time. This approach has not yet been applied in GC or GC-MS, and it can be considered as an improvement in HPLC analysis because it allows one to reach similar detection limits to those obtained in GC.

Conclusions

This work was focused on the development of an on-line procedure with pre- and post-column derivatization for determination of amines in biological samples. It is the first time that such an on-line procedure has been described.

The combination of pre-column derivatization with Dns-Cl by using a C_{18} packing material and multi-dimensional liquid chromatography coupled with post-column chemiluminescence generation with TCPO–H_2O_2 is a viable alternative to conventional solution derivatizations in the determination of primary and secondary amines. This study illustrates the potential of an automated system for on-line sample clean-up and sample treatment of amines in the analysis of biological samples before and after LC analysis. By using these procedures the determination of amphetamines can be performed with satisfactory sensitivity, reproducibility and a minimum of sample handling. The described approach is fully automatic, requiring only electrically controlled switching valves.

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