Journal of Chromatography, 593 (1992) 9–14
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2434

Determination of amphetamines by high-performance liquid chromatography with ultraviolet detection

On-line pre-column derivatization with 9-fluorenylmethyl chloroformate and preconcentration

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ABSTRACT

An on-line pre-column derivatization method for the determination of low concentrations of amphetamines using ultraviolet detection has been developed. In this work 9-fluorenylmethyl chloroformate (FMOC-Cl) has been used as the derivatizing agent. The optimum conditions for derivatization such as pH, reaction time and FMOC-Cl/amphetamine concentration ratio have been investigated. Attempts have been made to extend the sensitivity of the method by preconcentration of the derivatives on a micro-column packed with C_{18} bonded silica. Derivatization and preconcentration of the samples were carried out at low pressure on a flow injection analysis system. Quantitative determination of amphetamines as low as $2 \cdot 10^{-8}$ mol/l can be made using this on-line method with preconcentration. The sensitivity of this technique is about 50 times greater than the equivalent off-line method.

INTRODUCTION

Quantitative analysis of drugs such as amphetamine, methamphetamine, ephedrine, norephedrine and other amphetamine-related compounds which are used as stimulants has become important in clinical and forensic sciences.

Amphetamine and methamphetamine are the only drugs of this family which have been included in Swiss drug abuse legislation. However, stimulants such as ephedrine and norephedrine are banned by the International Olympic Committee because they are considered as dopants. Ephedrine and norephedrine in nose sprays are used as the starting products for amphetamine and methamphetamine synthesis [1].

Many analytical methods have been developed for their determination, including gas chromatography (GC) [2], thin-layer chromatography (TLC) [3] and high-performance liquid chromatography (HPLC) [4–6]. Biological samples often contain low concentrations of these drugs. Although GC and

0021-9673/92/$05.00

TLC are sensitive enough for their determination, they require time-consuming sample preparation. HPLC has the advantage that it is simple and aqueous samples, especially urine, may be analysed with a minimum of sample preparation.

The determination of amphetamines and amphetamine-related compounds by HPLC with UV detection has not gained much popularity due to the low absorbances of these compounds (molar absorptivity about 200 l cm^{-1} mol^{-1} at 257 nm in water).

Improvements in the detection limits is possible by using pre-column or post-column derivatization. In fact, a number of derivatizing agents such as *o*-phthalaldehyde (OPA), 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and sodium β-naphtaquinone-4-sulphonate (NQS) have been used to overcome the detection problem. These pre-column derivatization reagents have been used for the qualitative and quantitative analysis of amphetamines in urine and plasma samples [7].

The derivatizing agent 9-fluorenylmethyl chloroformate (FMOC-Cl) was first introduced for the derivatization of amino acids by Einarsson *et al.* [8]. It is suitable for the pre-column derivatization of primary and secondary amino acids [8,9] and amines [10,11]. The products formed with FMOC-Cl are stable, as opposed to those with OPA, which forms unstable derivatives and does not react with secondary amino acids.

As FMOC-Cl is a good derivatization agent for amines, it would be expected to be a good derivatizing agent for amphetamines, which also have amine groups. This was shown by Veuthey and Haerdi [12] and Gao *et al.* [13]. FMOC-Cl was therefore chosen as a derivatizing agent for amphetamines in this study.

The aim of this work was to develop an analytical method with on-line pre-column derivatization with FMOC-Cl for the determination of low concentrations of amphetamines using UV detection. The optimum conditions for derivatization such as pH, choice of solvent, reaction time and derivatization agent/amphetamine concentration ratio have been investigated. Attempts have been made to extend the sensitivity of the method by preconcentration of the derivatives on a micro-column packed with C_{18} bonded silica. Derivatization and preconcentration of the samples were carried out at low pressure by a flow injection analysis (FIA) system.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a Varian 5000 high-performance liquid chromatograph. An HP 1050 series variable-wavelength detector coupled to an HP 3390A integrator was used. A Gilson Minipuls-3 peristaltic pump and a Knauer 64 pump were used in the FIA system for the on-line derivatization and preconcentration steps. PTFE tubings (0.8 mm I.D.) were used in all instances. The reaction coil consisted of a glass tube (10 m × 2 mm I.D.). The FIA system was coupled to the HPLC system via a six-way Rheodyne 7000 valve coupled to a trace enrichment cartridge (pre-column), which is a cylindrical stainless-steel tube (13 mm × 2 mm I.D.) (Fig. 1) packed with 40–63-μm Nucleosil C_{18} silica (MN, Düren, Germany). The analytical column (200 mm × 4 mm I.D.) was packed with 5-μm Nucleosil C_{18} (MN).

Chemicals

Acetonitrile (HPLC grade) was obtained from Romil (Loughborough, UK). Distilled water was used for the preparation of the aqueous mobile phase. The mobile phase was filtered through a 0.45-μm Schleicher and Schuell membrane. FMOC-Cl, sodium carbonate (reagent grade) and sodium hydrogencarbonate (reagent grade) were obtained from Merck (Darmstadt, Germany), (−)-Ephedrine and D-(+)-norephedrine from Fluka Chemie (Buchs, Switzerland), DL-amphetamine sulphate from Siegfried (Zofingen, Switzerland) and DL-methamphetamine hydrochloride from the Federal Office of Public Health (Switzerland) were used. Stock solutions (0.01 mol/l) of each of the amphetamines in 0.1 mol/l hydrochloric acid were stored at

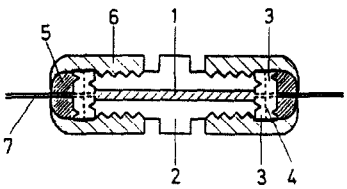


Fig. 1. Enrichment cartridge. 1 = Internal volume (13 mm × 2 mm I.D.) packed with 40–63 μm Nucleosil C_{18} silica; 2 = pre-column; (3) PTFE O-ring; 4 = sintered stainless steel (porosity 20 μm); 5 = adaptor for sealed capillary; 6 = nut "Serto"; 7 = stainless-steel capillary.

5°C. A stock solution (0.01 mol/l) of FMOC-Cl in acetonitrile was prepared. Sodium hydrogencarbonate–sodium carbonate (0.1 mol/l) buffer solution (pH 9.0) was prepared for the off-line derivatization. For the on-line derivatization the concentration of the buffer was 0.2 mol/l.

Procedure

Off-line derivatization. To 35 ml of 0.1 mol/l carbonate buffer (pH 9.0) placed in a 50-ml volumetric flask, 5 ml of an aqueous solution containing the four amphetamines ($1 \cdot 10^{-6}$–$1 \cdot 10^{-4}$ mol/l) and 10 ml of $5 \cdot 10^{-4}$ mol/l FMOC-Cl in acetonitrile were added and diluted to the mark with carbonate buffer. After a 10-min reaction time, 200 µl of the mixture were injected into the HPLC system for separation.

On-line derivatization and preconcentration. A schematic diagram of the system used is shown in Fig. 2. The sample solution was pumped by a peristaltic pump to tee 1 (6) where it was mixed with the carbonate buffer. Switch valve 4 was used for pumping $5 \cdot 10^{-5}$ mol/l FMOC-Cl in acetonitrile to tee 2 (7). FMOC-Cl and the sample solutions were pumped simultaneously. Once the desired volume of sample solution had been pumped, the six-port valve 3 was switched to pump water for the clean-up of the tubes. Valve 4 was switched 1 min after switching valve 3 to allow the FMOC-Cl solution to circulate back into the reservoir. Before linking the pre-column to the analytical column for the separation of the derivatized products, using valve 5, the glass coil and the pre-column were flushed with the pumping solution, the volume used being slightly greater than the capacity of the coil (31.4 ml).

It is important to note that preliminary tests using a PTFE or nylon reaction coil showed that these coils were unsuitable as a result of adsorption problems and only glass reaction coils gave satisfactory results.

RESULTS AND DISCUSSION

Amphetamines react with FMOC-Cl under alkaline conditions to form amino derivatives. In addition, FMOC-Cl undergoes hydrolysis to produce FMOC-OH. These reactions are shown in Fig. 3. The derivatization and hydrolysis reactions are influenced by factors such as pH, FMOC-Cl/amphetamine ratio and derivatization time. The effect of these parameters was studied by the batch method using HPLC.

The spectral characteristics of the derivatives is important in optimizing the sensitivity of the detector. Therefore, the UV spectra of the derivatized amphetamines and the hydrolysed FMOC-Cl (FMOC-OH) were run. The spectra of derivatized amphetamines and FMOC-OH show absorption maxima at 208 and 265 nm (Fig. 4). Although the absorbance at 208 nm is much higher than at 265 nm, 265 nm was chosen for these measurements as at 208 nm the absorbances due to the eluent (acetonitrile–water) and interfering substances in the test solution were fairly high.

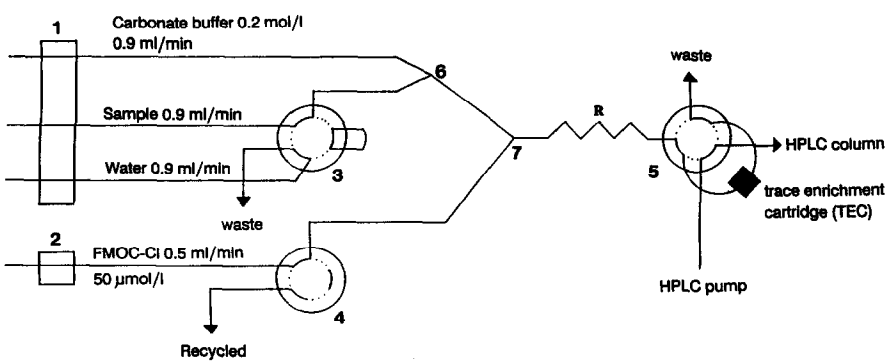
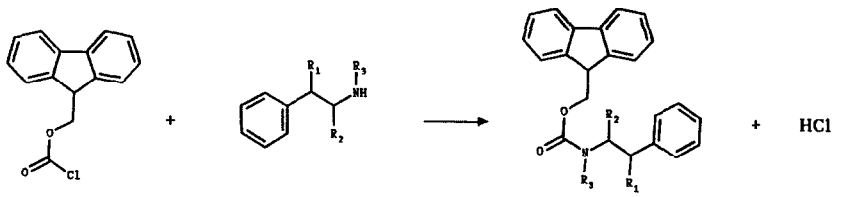


Fig. 2. Schematic diagram of the system used for on-line preconcentration and derivatization. 1, 2 = Pumps; 3, 4, 5 = switch valves; 6 = tee 1; 7 = tee 2; R = glass coil (capacity 31.4 ml).

PRIMARY REACTION :



	R_1	R_2	R_3
Amphetamine	-H	$-CH_3$	-H
Methamphetamine	-H	$-CH_3$	$-CH_3$
Ephedrine	-OH	$-CH_3$	$-CH_3$
Norephedrine	-OH	$-CH_3$	-H

SECONDARY REACTION :

$$\text{FMOC - Cl} \xrightarrow{OH^-} \text{FMOC-OH}$$

Fig. 3. Reaction between FMOC-Cl and amphetamines under alkaline conditions.

Effect of pH

The optimum pH for derivatization was between 9 and 10. For pH > 10, the hydrolysis of FMOC-Cl is considerably higher than at lower pH values. In addition, the silica degrades at this pH. For pH < 9 the derivatization time for amphetamines was too long. Thus pH 9 was chosen for derivatization and a carbonate buffer was used to maintain this constant pH.

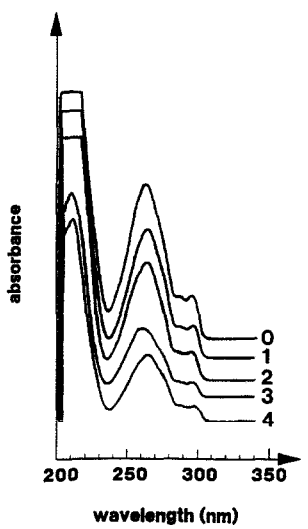


Fig. 4. Spectra of the derivatized amphetamines and FMOC-OH. 0 = FMOC-OH; 1 = methamphetamine; 2 = ephedrine; 3 = norephedrine; 4 = amphetamine.

Stability of the derivatives

The derivatives remained stable for 1 week if they were stored at 5°C.

Effect of FMOC-Cl/amphetamine ratio

As the hydrolysis of FMOC-Cl depends on its concentration, measurements were made keeping its initial concentration constant and varying the amphetamine concentration. The derivatization of amphetamine was independent of the FMOC-Cl/amphetamine ratio when this ratio was between 10 and 1000.

Optimum reaction time

A preliminary study of the reaction time indicated that for reaction times greater than or equal to 10 min, peak areas were independent of time for all

the amphetamines tested. Thus, for the on-line derivatization reaction, the capacity of the reaction coil was chosen such that the reaction time is greater than 10 min for the flow-rates used in this work.

Separation of amphetamines

Amphetamines were separated by HPLC using the optimum conditions of flow-rate, 1.5 ml/min, and eluent acetonitrile–water (58:42, v/v). A typical chromatogram obtained for the separation of amphetamines from a solution containing four different amphetamines is shown in Fig. 5 (off-line method). The peak at 42.13 min is probably the carbonic acid ester of FMOC, a product resulting from the condensation reaction between FMOC-Cl and FMOC-OH. UV spectra do not discriminate between this compound and FMOC-Cl.

Analogous chromatograms were obtained for on-line derivatized and preconcentrated samples.

Breakthrough volume

To determine the loading capacity of the pre-column, a solution containing the four different amphetamines ($1 \cdot 10^{-5}$ mol/l each) was derivatized by the off-line method and passed through the column. Aliquots of the mixture were collected every 5 min at the outlet of the column and injected into the HPLC system for separation. The volume of solution passed through the column may be computed from the flow-rate and the time of passage. The breakthrough volume was 45 ml using this procedure.

Calibration graph

Off-line method. Linear calibration graphs were obtained over the range $1 \cdot 10^{-6}$–$1 \cdot 10^{-4}$ mol/l for each of the amphetamines corresponding to $2 \cdot 10^{-11}$–$2 \cdot 10^{-9}$ mol injected, the correlation coefficient being greater than 0.999.

The reproducibility of the injections was tested by making five replicate measurements. The results showed that the reproducibility at low concentrations was 10% and that for high concentrations it never exceeded 2%.

On-line method. Linear graphs were obtained in the range $2 \cdot 10^{-8}$–$1 \cdot 10^{-7}$ mol/l for each of the amphetamines. Correlation coefficients (r) for methamphetamine and norephedrine were 0.979 and 0.958, respectively, whereas for the other two substances they were greater than 0.997. Despite the fact that the r values for methamphetamine and norephedrine are not very good, they are acceptable for quantification considering the low levels of these substances. A comparison of these results with those obtained by the off-line method shows that the sensitivity of the method is increased by a factor

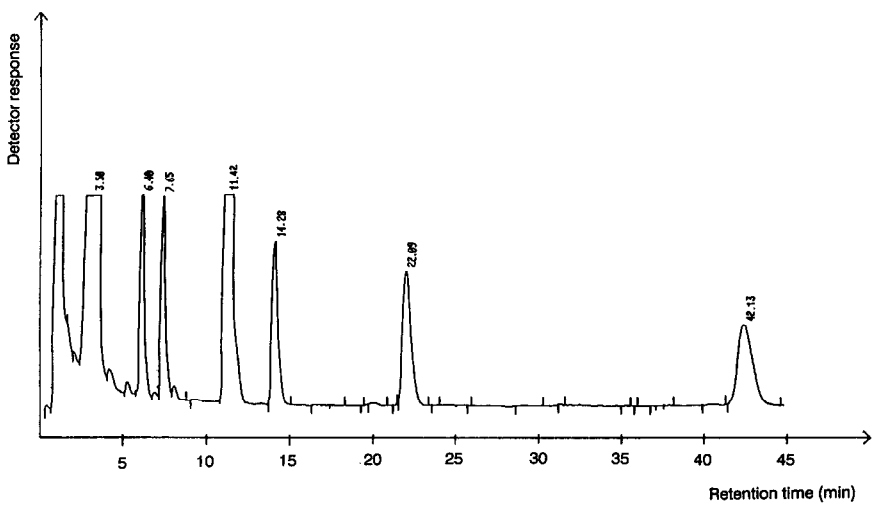


Fig. 5. Typical chromatogram obtained for a mixture of four derivatized amphetamines (off-line method). The injected amounts ($2 \cdot 10^{-10}$ mol) were the same for all the amphetamines. Retention times (min): FMOC-OH, 3.50; norephedrine, 6.40; ephedrine, 7.65; FMOC-Cl, 11.42; amphetamine, 14.28; methamphetamine, 22.09; $(FMOC)_2$ carbonic acid ester, 42.13.

of about 50. The reproducibility for methamphetamine and norephedrine was 13.5 and 16.1%, respectively, whereas for ephedrine and amphetamine it was 4.8 and 5.0%, respectively. Better reproducibilities, particularly for methamphetamine and norephedrine, may be achieved by making slight alterations to the experimental procedure.

CONCLUSION

The results of this study have shown that amphetamines may be determined quantitatively using FMOC-Cl as a prederivatizing agent. Good separation of the amphetamines was observed. In contrast to the batch method, the on-line prederivatization–preconcentration method enhanced the sensitivity of the analytical determination. The application of the method to biological samples is currently under investigation.

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