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Use of 2-chloroethylnitrosourea, a new type of pre-column derivatizing agent for the measurement of biogenic amines, by high-performance liquid chromatography with ultraviolet detection

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Abstract

A new type of derivatizing agent 2-chloroethylnitrosourea (CENU) has been developed for biogenic amine determination. The compounds of this family of reagents have a modular structure which allows modification in the type of detection. The derivatization reaction takes 15 min at about 75°C and is brought about by mixing a basic aqueous solution of an amine (histamine, cadaverine, putrescine) with an acetonitrile solution of 2-chloroethylnitrosourea (CENU). This reaction is simple, fast and complete. The derivative obtained is very stable (more than ten days). The selectivity and efficiency of the reaction of CENU on primary amines contribute to the high sensitivity of the method which is thus adapted to the measurement of very low concentrations of biogenic amines. © 1998 Elsevier Science B.V.

Keywords: Derivatization, LC; 2-Chloroethylnitrosourea; Histamine; Cadaverine; Putrescine

1. Introduction

Histamine is a biogenic amine present in variable concentration in foods such as fish, shellfish, wine and cheese, as well as in biological tissues. Biogenic amines are formed by the decarboxylation of certain amino acids. These molecules are currently recognized as indicators of food quality. The measurement of trace levels of biogenic amines requires a specific and sensitive technique [1]. Most of these compounds cannot be detected as they lack a suitable chromophore, whereas others such as histamine are detected at wavelengths that are not selective. In order to prevent matrix interference and so enhance detection sensitivity when using HPLC, it is necessary to resort to derivatization techniques [2]. The derivative obtained is detected using ultraviolet or more commonly fluorescence techniques [3].

OPA (*o*-phthaldialdehyde) is the derivatizing agent most commonly used for amino compound determination [3–14]. However other derivatizing agents have been described such as dansyl chloride [15–18], fluorescamine [19], fluorenylmethoxycarbonyl [2,20,21], fluorenacetyl and benzoyl chloride [1,22,23]. Most of these reagents give unstable derivatives. In the case of OPA both the reagent and the derivative are unstable [3,10–12,14,19,25]. A number of authors have recently reported on their

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studies of the effect of the derivatization parameters on the stability of OPA derivatives [3,10,11,14, 25,26]. These all point to the importance of derivative stability in amino compound determination. Pawlowska et al. [24] have shown that the derivatives obtained with aromatic anhydrides are in this respect superior to those obtained using OPA. In addition the derivatization is fast compared to that using benzoyl chloride or dansyl chloride.

Post-column derivatization is usually used with reagents giving unstable derivatives. These methods are more complicated than pre-column methods due to the complex and expensive equipment required for the derivatization [27]. The peaks are broadened and diluted by the derivatization reagents adding a further inconvenience [3,25]. Pre-column derivatization is widely used [2,5–8,15,20–22,28,29]. This method is very flexible to use [2] providing derivatives are stable [19], otherwise it is necessary to rigorously control the time and conditions of the derivatization reaction [3,10,14,19]. On-line derivatization [3,12,14] allows continuous analysis but limits the choice of the labeling reagents. Moreover this method requires an expensive set-up in order to optimize the chromatographic conditions [19]. Nevertheless K. Saito et al. have reported that the stability of the derivatives is increased with this derivatization method [3].

We required a method which would allow a rapid quantization of amines with a high degree of simplicity and decided that the pre-column derivatization would be the most appropriate method to use. The search for a new derivatizing agent satisfying the need for derivative stability and for method sensitivity led us to an interest in CENUs (Fig. 1). CENU's are studied as active antitumor agents. The compounds decompose rapidly under physiological conditions producing alkylant and carbamoylant moieties [30]. Carbamoylation is thought to be caused by organic isocyanates on proteins [31]. Isocyanates are known to react with molecules containing primary amines to form a stable asymmetric urea [32]. Such derivatives are obtained with 6 aminoquinolyl-*N*-hydroxysuccinimidyl carbamate [32–34].

This article presents the use of CENUs described in Fig. 2 (a and b) as pre-column derivatizing agents of amines followed by HPLC separation with UV detection.

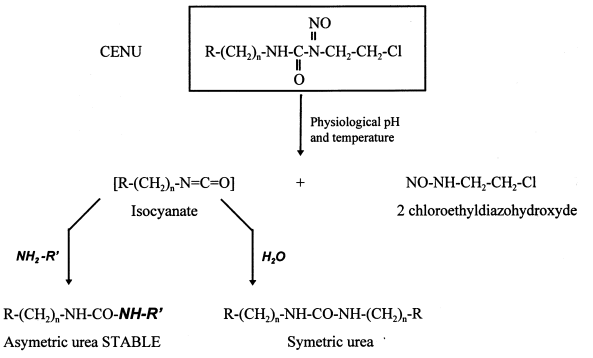


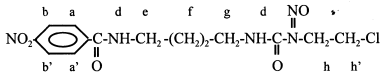
Fig. 1. CENU's structure and reactivity.

2. Experimental

2.1. Apparatus

The HPLC system is from TSP (Thermo Separation Product, les Ulis France) and consists of a P4000 pump system, an SN 4000 interface, an AS 3000 turnable sampler, and a UV spectra Focus detector. The detector allows UV spectra to be taken for the whole period of the chromatogram. During the design phase of the method, the data acquisition

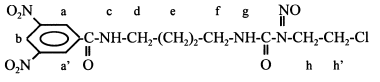
a). CENU-NO_2 : (melting point : 131-132 °C)



N-pNitrobenzoyl N'[N-(2chloroethyl)-N-nitroso] carbamoyl-1,4-diaminobutane

δppm/TMS (DMSO-d6) : 1.6 (t, 4H, CH_2f); 3.3 (m, 4H, CH_2h,h'); 3.6 (t, 2H, CH_2g); 4.1 (t, 2H, CH_2e); 8.05 (m, 2H, Ha, Ha'); 8.3 (m, 2H, Hb, Hb'); 8.8 (t, 2H, 2NHd).

b). CENU-2NO_2 : (melting point : 132 °C)



N-2,4 Dinitrobenzoyl N'[N-(2chloroethyl)-N-nitroso] carbamoyl-1,4-diaminobutane

δppm/TMS (Aceton-d6) : 1.8 (t, 4H, CH_2e); 3.55 (m, 6H, CH_2f,h,h'); 4.15 (t, 2H, CH_2d); 8.1 (t, 1H, NHg); 8.55 (t, 1H, NHc); 9.1 (m, 3H, Ha,a',b).

Fig. 2. Structure of the CENU used for the amine derivatization.

was done by sweeping the spectrum between 221 and 301 nm every 2 nm, which corresponds to a point every 0.5 s. The spectrum of molecules was stored in a library and serves as a reference for the identification of spectra acquired in the same chromatographic conditions. This also allows information on the purity of the detected peak. After the first design phase, all the data acquisition was made in single wavelength at 271 nm for CENU-NO_2 (Fig. 3) (and at 231 nm for CENU-$2NO_2$). The UV spectrum in Fig. 3 corresponds to the spectrum of the chromophore part of the CENU-NO_2 without the N-NO-CH_2-CH_2-Cl part which is a leaving group not implicated in the derivative detection.

The analytical column was a Shandon RP C_{18} 5 μm, 250×4.6 I.D. (Shandon Eragny sur Oise, France).

An ultrasonic Baudelin Sondex RK 100 bath was used for the derivatizations. A hydrogen NMR (Bruker AC 250, 250 MHz) was used to study the structure of the standards.

2.2. Reagents

Histamine dihydrochloride, putrescine and cadaverine were purchased from Aldrich (St Quentin Fallavier, France). Acetonitrile, acetic acid (Carlo Erba, Nantaire France) and ammonium acetate (Merk, Darmstardt, Germany) were used to prepare the mobile phase. Pure water was produced by a Millipore Milli-Q Plus System (St. Quentin, Yvelines, France). Chromatographic runs were carried out either using a binary isocratic or binary gradient.

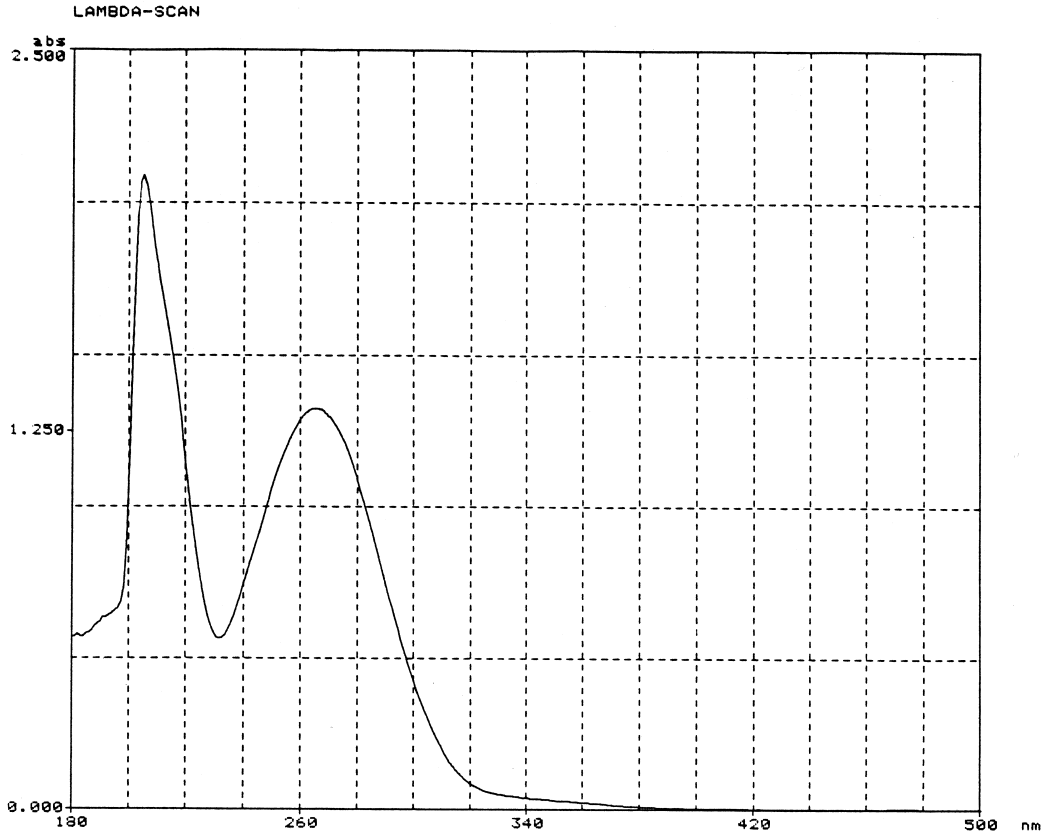


Fig. 3. UV spectrum of the chromophore part of CENU-NO_2 without the leaving group (N-NO-CH_2-CH_2-Cl). 1.4 *M* in acetonitrile.

Ammonium acetate buffer (0.1 *M*) was prepared by dissolving ammonium acetate in water and titrating to pH 6 with acetic acid.

Mobile phases were: A, water 0.5 m*M* ammonium acetate buffer, and B, acetonitrile–water–0.5 m*M* ammonium acetate buffer (80:20, v/v).

The column was maintained at 40°C and the flow-rate was 0.8 ml min^{-1} for all chromatographic separations.

All chemicals were used as received without further purification.

2.3. Synthesis of derivatization reagent and amine standards

CENU (as in Fig. 2) was synthesized following the protocol described by J. Martinez et al. [35]. CENU NO_2 and $2NO_2$ are novel. Their physical properties are given in Fig. 2 (NMR and melting point).

The production of labeled-amine standards was assured by mixing a solution of CENU (508 mg of CENU in 10 ml of acetonitrile) with an excess of amine (1.8 g of histamine dihydrochloride or 277 mg of putrescine or cadaverine dissolved in 10 ml of water). The pH was adjusted to 10 with 10 *M* sodium hydroxide solution (NaOH). The mixture was then shaken in an ultrasound bath at 60°C for 4 h. The acetonitrile was evaporated under reduced pressure. The precipitate obtained was filtered. The purity of the standards thus produced was controlled by NMR and HPLC. The derivatized amines produced were in solid form and could be used as standards.

2.4. Derivatization principle

The principle of the derivatization of amines is equivalent to that of the production of standards. The amine is dissolved in 10 ml of water and added to a solution of 10 ml of CENU in acetonitrile. In this case the derivatization reagent is in excess in comparison with the amine whereas in the case of the production of standards it is the opposite. The pH was adjusted to 10 with 10 *M* NaOH, then the mixture was shaken in a thermostatically controlled ultrasound bath.

2.5. Assessment method of the influence of reaction parameters on the level of derivatization

The influence of four parameters – pH, temperature, time and CENU/histamine ratio – on the effectiveness of the derivatization was studied by simultaneously varying the different parameters using the factorial matrix principle. Two levels, maximum and minimum, were tested for each parameter. The choice of minimum and maximum levels was made with the help of preliminary tests results (which are not detailed in this paper). There was evidence suggesting that pH and CENU/histamine ratio have a strong influence on the percentage of derivatization, whereas temperature and time did not seem to modify the coupling outputs significantly.

In the case of the pH the results between pH 9 and 10 were similar, but the level of derivatization went down below pH 8 and above pH 11.

Table 1 gives the values of the parameters for each experiment.

The derivatization performance is calculated from the values of linear regressions obtained over a range of standard concentrations between 0.1 and 100 ppm. A labeled amine stock solution (1 g l^{-1}) is prepared in water–acetonitrile at pH 10 (50:50, v/v). This solution is then diluted (in order to obtain the different concentrations) in a mixture of water–acetonitrile pH 10 (50:50, v/v). 10 μl of each standard solution are injected, which corresponds to 0.2–29.5 ng of histamine deposited on the column.

The histamine derivatization was first studied using the technique described here. The feasibility of this method (with parameters thus determined) on

Table 1
Parameters values for the different experiments

Experiment number	pH	Temperature (°C)	Min	CENU/histamine
1	9	60	15	4
2	10	60	15	6
3	9	75	15	6
4	10	75	15	4
5	9	60	60	6
6	10	60	60	4
7	9	75	60	4
8	10	75	60	6

putrescine and cadaverine will be presented afterwards.

3. Results

3.1. Histamine

3.1.1. Linearity and detection limits

The linearity of detection was assessed between 0.1 and 100 mg l^{-1} of histamine derivative (UH) standards (8 points: 0.1, 0.4, 0.5, 2, 5, 10, 25, 100 ppm).

For a simple and rapid determination of histamine, the elution was done using an isocratic (0 to 10 min: 68% A, 32% B at 40°C 0.8 ml min^{-1}). The linearity of the detection is excellent between 0.1 and 100 ppm (10 μl injected) with a linear regression coefficient (R^2) of more than 0.999. The regression equation $Y=aX+b$, where X is the concentration of UH (ppm) and Y the peak height, is $Y=891.1X-239.5$. The asymmetry factor (at 10% of the height) is between 1 and 2 depending on the concentrations, and the HEPT is 0.05 mm.

The relative standard deviation (R.S.D.) for the peak height ($n\geq 3$) over the range of concentrations tested was never more than 7.3% and for the retention time ($n=38$) never more than 0.76%.

When histamine is derivatized, the CENU excess reacts with water (Fig. 1) and gives a degradation compound (Ud) which is eluted in 3.97 min. UH is eluted in 5.07 min. The isocratic elution used thus permits an effective and rapid separation of the different compounds present in the solution (Fig. 4).

The detection limit is 8.4 pmol of injected histamine with a signal to noise ratio of 3:1.

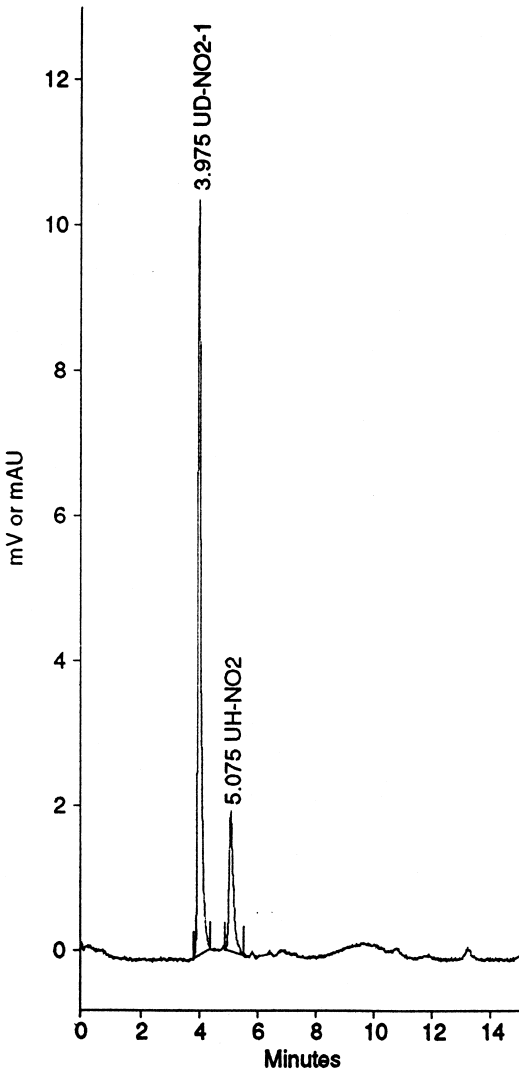


Fig. 4. Typical chromatogram of histamine determination. Peaks: UD–NO_2=CENU degradation product, UH–NO_2=CENU–histamine derivative. Experimental conditions are: 68% A (water–0.5 m*M* ammonium acetate buffer), 32% B (acetonitrile–water–0.5 m*M* ammonium acetate buffer; 80/20, v/v); 0.8 ml min^{-1}, C_{18} 5 μm, 250×4.6 mm I.D. maintained at 40°C. The *LD*=8.4 pmol (signal/noise=3).

3.2. Derivatization optimization

The results obtained with the factorial fraction matrix experimental plan (Table 1) are presented in Table 2. All experiments were carried out twice and each sample injected twice. The results clearly showed that the derivatization is reproducible equally well at the coupling and at the detection stages. The variation coefficients for each experiment are less than 1%. The equation of the derivatization rate DR as a function of the 4 parameters thus obtained is:

$$DR = 81.666 + 5.61X_1 + 3.93X_2 + 3.89X_3 - 0.72X_1X_2 - 1.0125X_1X_3 - 2.49X_2X_3 - 3.62X_1X_2X_3 .$$

with X_1=pH, X_2=temperature, X_3=reaction time, X_1X_2=pH–temperature interaction, X_1X_3=pH re-

Table 2
Derivatization rate obtained with experiments presented in Table 1 for histamine

No.	Derivatization rate (%) 1st and 2nd injection	Derivatization rate (%) 1st and 2nd injection	Mean derivatization rate (%)	S.D.[a]	R.S.D.[b] (%)
1	67.70	68.21	67.63	0.60	0.89
	66.62	67.97			
2	75.64	75.45	75.06	0.52	0.69
	74.32	74.83			
3	70.46	79.62	74.71	3.7	4.9
	71.87	76.9			
4	93.79	93.86	93.71	0.35	0.37
	93.13	94.07			
5	71.31	78.5	75.17	3.85	
	73.5	77.38			
6	93.65	92.85	93.07	0.38	0.41
	93.15	92.62			
7	86.18	86.9	86.73	0.62	0.72
	87.69	86.16			
8	86.26	87.25	87.25	0.79	
	87.69	88.23			

[a] Standard deviation.
[b] Relative standard deviation.

action time interaction, X_2X_3 = temperature–reaction time interaction, $X_1X_2X_3 = X_4$ = ratio CENU/histamine.

The parameters studied seem to have a significant influence on the output of derivatization. The output equation shows that the pH must be at the maximum level (pH 10). The reaction time and temperature should not be simultaneously at their minimum (Table 3). Table 3 shows that in this case the calculated outputs are identical for other combinations of these two parameters. This means that the reaction is complete after 15 min at 75°C and that it will not develop further for 45 min. At 60°C it takes about 45 min to obtain the same result.

Table 3
Derivatization rate calculated for each time and temperature combination, with all others parameters constant

Temperature (°C)	Time (min)	Derivatization rate (%)
Maximum (75°C)	Maximum (60)	94.48
Maximum (75°C)	Minimum (15)	93.71
Minimum (60°C)	Maximum (60)	93.07
Minimum (60°C)	Minimum (15)	82.29

The pH–temperature interaction and the pH–reaction time interaction are negligible.

Nine CENU/histamine ratios between 2 and 114 were tested. The concentration of histamine was fixed at 100 ppm. The level of derivatization was always within the range of 84% to 123.6%; no significant differences appeared between the nine CENU/histamine ratios. The mean level of derivatization calculated ($n = 42$) is 100.3% ±14.2%.

The results obtained with the experiment plan seem to indicate that with a ratio CENU/histamine of 2, the output is better than with a ratio of 4. This is in contradiction with the precedent results and the ratios tested in the experience plan (2 and 4) are not sufficient for the quantification of an unknown concentration of histamine. The rest of the work was carried out using a minimum CENU/histamine ratio of 8.

3.3. Derivatization rate and histamine derivative stability

Increasing concentrations of histamine (between

18–270 μmol l^{-1}) were derivatized in the same conditions: 15 min, pH 10, 75°C, with a minimum ratio of CENU/histamine of 8. The CENU solution used is the same for each sample of histamine, consequently the CENU/histamine ratio increases as the histamine concentration decreases. Each experiment was repeated twice. After derivatization, the samples were immediately injected, kept for two days in daylight and at ambient temperature, then stored for 20 days in a refrigerator at 4°C.

The results presented in Table 4 show that the level of derivatization is constant and repeatable whatever the concentration of histamine. This confirms that the ratio CENU/histamine has no effect. The mean output is therefore 101.9%±7.07. In order to ensure the stability of the derivatives, the samples were reinjected 22 days later. The derivative concentration was recalculated (the calibration was done for each series of injections). The results obtained after 22 days enabled a comparison to be made with the initial results by calculating the derivatization output. It appears clear that the derivative remains stable for 22 days without taking any particular precaution concerning exposure to light (Student's test $\alpha < 0.001$).

3.4. Modulation of the chromophore intended to improve detection limits

In order to increase the sensitivity of the method, a CENU carrying a different chromophore was tested. This new molecule (CENU–$2NO_2$) was no different than the previous one except for the addition of an NO_2 group (Fig. 2b). A standard was produced using the method already described. The linearity observed between 0–100 ppm (0.0 at 26 μg of injected histamine) was excellent with a correlation coefficient (R^2) higher than 0.999. The regression of the peak height Y (CENU–$2NO_2$) as a function of the concentration X of CENU–$2NO_2$ in mg l^{-1} is $Y = 1129.46X + 32.64$. In this case, with the same isocratic as previously, the Ud–$2NO_2$ is eluted at 5.2 min and the UH–$2NO_2$ at 7.4 min (Fig. 5). The new molecule brings about a slightly less rapid detection than previously but with a better resolution R_s of the peaks ($R_{s_{NO_2}} = 4$ and $R_{s2_{NO_2}} = 6.54$ with $R_s = 2(t_{r2} - t_{r1})/(\omega_1 + \omega_2)$ where t_{r2} and t_{r1} are the peak retention times and ω_1 and ω_2, the widths of the peaks obtained by the tangent methods). The limit of detection calculated for a signal-to-noise ratio of 3:1 is 0.18 pmol for 10 μl injected. Detection using this new derivative agent is 43 times more sensitive. This difference could be explained by the molar adsorptivity of the two compounds (ε CENU–NO_2 (methanol) = 11558 $dm^3\,mol^{-1}\,cm^{-1}$ at 271 nm and ε CENU–$2NO_2$ (methanol) = 20914 $dm^3\,mol^{-1}\,cm^{-1}$ at 231 nm), and by a higher column efficiency with CENU–$2NO_2$ derivatives with a reduced background noise.

3.5. Derivatization rate with CENU–$2NO_2$

We have verified that the reactivity of the CENU–$2NO_2$ was not affected by a change in the chromophore part. To do this we studied the coupling output

Table 4
Histamine derivatization rate results

Initial [histamine] μ*M* and (CENU/histamine ratio)	Derivatization rate (%) at $T = 0$	Derivatization rate (%) after 22 days
18 (120)	102.59	101.95
	106.81	108.43
45 (48)	90.31	97.23
	89.06	95.13
135 (16)	103.82	111.97
	108.85	114.35
270 (8)	107.58	107.92
	105.85	116.31
Mean (%)	101.9±7.07	106.6±6.78
R.S.D.	6.9%	6.3%

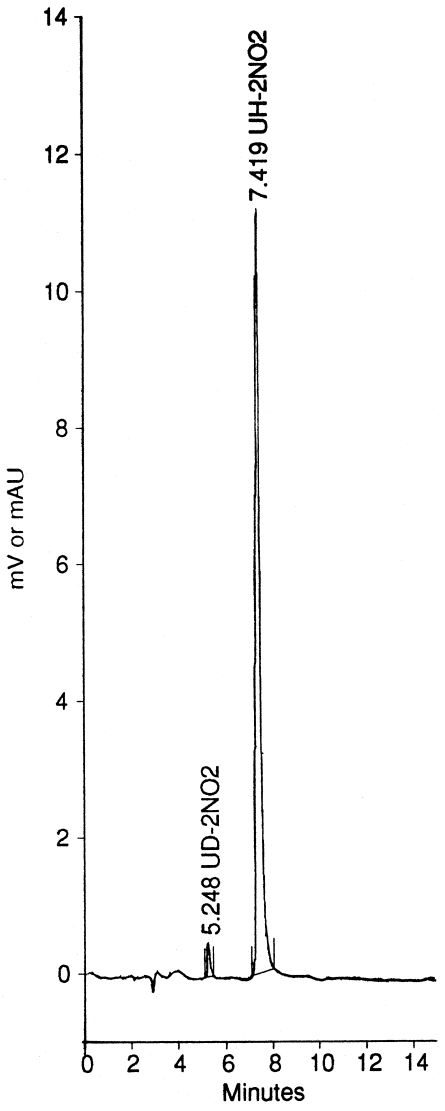


Fig. 5. Typical chromatogram of histamine determination with CENU–$2NO_2$ as derivatising reagent. Peaks: UD–$2NO_2$ = CENU degradation product, UH–$2NO_2$ = CENU–histamine derivative. Experimental conditions are: 68% A (water–0.5 m*M* ammonium acetate buffer), 32% B (acetonitrile–water–0.5 m*M* ammonium acetate buffer; 80/20, v/v); 0.8 ml min^{-1}, C_{18} 5 μm, 250×4.6 mm I.D. maintained at 40°C. The *LD* = 0.18 pmol (signal/noise = 3).

over a scale of histamine concentrations under exactly the same conditions as those used for CENU–NO_2.

The mean output calculated for CENU–$2NO_2$ is 101.71±18.4%. The stability of the new derivative was tested after 7 days (2 days at daylight and ambient temperature and 5 days at 4°C in the dark). After seven days the output measured was 98.4%±19%. This result is identical to that obtained at the time of the measurement immediately after derivatization (Student's test $\alpha < 0.001$). The properties of the CENU (affinity of the reagent and stability of the derivatives) are not changed by a modification of the chromophore.

3.6. Application of the method for the determination of putrescine and cadaverine with CENU–NO_2

3.6.1. Chromatographic separation and linearity of the detection

The separation of the three amines has been realised using a simple linear gradient ($t = 0$: 80%A–20% B; $t = 20$: 82%A–18%B). The putrescine derivative (UP) was eluted in 17.4 min, UH in 19.664 min and the cadaverine derivative (UC) in 21.035 min. The resolution factors R_s calculated on this gradient are R_s(Ud–UP) = 9.9; R_s(UP − UH) = 2.2; R_s(UH − UC) = 1.57.

The linearity was verified on a scale of concentration of UP and UC between 0 and 50 mg l^{-1} which corresponds to 0–1.42 nmol of injected putrescine or 0–1.36 nmol of injected cadaverine. The regression equation of the relative peak height (*Y*) versus the amount of labeled amine injected (*X*) is *Y*(UP) = 359.74 *X*(UP) + 181.76 and *Y*(UC) = 302.78 *X*(UC) − 78.46 with a correlation coefficient of more than 0.9999 in both cases. The detection limit calculated with a signal-to-noise ratio of 3:1 is 2.2 pmol injected for putrescine and 9.11 pmol injected for cadaverine.

3.7. Derivatization output and derivative stability

The derivatization output was calculated on increasing concentrations of amines (in a similar manner to that described for the histamine) using the previous parameters (pH 10, 75°C 15–60 min, CENU/histamine ≥8).

3.7.1. Results with putrescine

The results presented in Table 5 show a strong replicability (variation coefficient less than 5%). The

Table 5
Derivatization rate for putrescine

[Putrescine] (m*M*)	Derivatization rate (%) at $T=0$	Derivatization rate (%) after 14 days
0.022	97.2	89.7
	103.1	96.4
0.056	106.9	100.9
	114.0	106.5
0.113	106.2	100.1
	112.0	104.77
0.17	104.0	97.7
	108.8	101.3
0.34	104.3	100.31
	106.1	105.1
Mean (%)	106.26±4.7	100.26±4.9
R.S.D.	4.4%	4.88%

mean output of derivatization calculated (106%) for the analysis done immediately after treatment is within the confidence interval established for histamine. After 14 days, the mean output is in the region of 100% when it was 106%. A Student's test showed that this difference is significant with a risk α of 5%. This tendency is the opposite to that of histamine. The stability over this 14 day period has to be defined.

Putrescine is a symmetrical diamine which can, in theory, form two compounds by derivatization: a mono-derivative molecule and a double-derivative molecule. In principle these two molecules do not have the same chromatographic behavior. However a single peak only has been observed on chromatogram. Furthermore in the standard preparation stage a precipitate formed (in particular conditions) and was identified by NMR as the double-substitute amine. This insoluble (in acetonitrile–water, 1:1) double-substitute amine is therefore not measurable during HPLC analysis. It is therefore plausible to suggest that the form detected with an output of 100% is the mono-derivative putrescine. Currently the mechanism of this reaction is not precisely known, but it would be very interesting to study it.

3.7.2. Results with cadaverine

The results are presented in Table 6. The mean output calculated immediately after derivatization is 50.87% with a weak variation coefficient. The derivatization output obtained whatever the concentration of CENU is surprising. Cadaverine shows two

Table 6
Derivatization rate for cadaverine

[Cadaverine] (m*M*)	Derivatization rate (%) at $T=0$	Derivatization rate (%) after 14 days
0.020	50.16	50.02
	53.27	52.69
0.052	45.47	48.34
	50.14	49.6
0.104	48.67	53.24
	54.59	53.96
0.156	52.25	51.71
	52.6	52.43
0.313	54.84	54.03
	50.17	49.99
Mean (%)	50.0±2.58	51.60±1.99
R.S.D.	5.08%	3.86%

symmetrical amine functions. As mentioned above, there are, therefore, in theory, two different derivatized molecules. As with putrescine, the double-derivative compound identified by NMR proved to be insoluble in the reaction medium. The compound detected with HPLC would therefore be the mono-substitute cadaverine. The results seem to show that in the derivatization conditions studied 50% of the molecules are not derivatized or are derivatized twice.

During the phase where the synthesis of standards was perfected, the disubstitute form of putrescine was obtained under certain conditions, while in the derivatization condition this compound has not been formed. This shows that the formation of the double-derivative compound depends more on the derivatization conditions than on the tridimensional conformation of the amine. It should be the same for cadaverine.

In the derivatization condition of pH, 50% of cadaverine is protonated twice and 50% protonated once ($pK_1 \approx 10.96$, $pK_2 \approx 10.02$). The most probable reactive form of the amine seems to be the non-protonated form. The level of derivatization with histamine and putrescine (100%) in the derivatization conditions used is in accordance with their basicity (histamine: $pK_1 \approx 9.68$, $pK_2 \approx 5.88$; Putrescine: $pK_1 \approx 10.8$, $pK_2 \approx 9.35$).

The cadaverine derivative is very stable (identical results after 14 days according to Student's test).

4. Discussion

The purpose of this study was to establish a simple and reliable method of quantifying biogenic amines and in particular to provide an improvement in the stability of the derivatives compared to existing methods.

The first thing studied concerned the feasibility of detection as well as its characteristics of specificity and linearity. The UV spectrum of the CENU–NO_2 chromophore part (see Fig. 3) shows two absorption maxima, one at 210 nm and the other at 271 nm. The 271 maximum allows a more specific detection with the least background noise. The detector response at 271 nm is linear between 0 and 100 ppm histamine ($R^2 > 0.999$). The peak is well defined with an isocratic elution which allows relatively short chromatogram runs. In these respects, this method is equivalent to those referred to the literature.

The later stage of this study shows that the level of derivatization is nearly 100%. This rate of derivatization is reached after 15 min at 75°C. The conditions for the derivatization are easy since the only strict constraint is that a pH of 10 is required. It is not worth rigorously controlling the reaction time since at 75°C this will be between 15 to 60 min. Equally, it is possible to lower the temperature if the reaction time is increased (60 min at 60°C is equivalent to 15 min at 75°C). This flexibility is much more superior to classical methods in which the reaction time is critical due to the instability of the derivatives [3,14,19]. It is thus easy to carry out pre-column derivatization.

With other methods, the need to rigorously respect the derivatization conditions is a constraint. In order to improve the replicability, researchers [3,12,14,20,36] have perfected new line chromatographic methods, but these, unfortunately, require further investment. The methods are particularly difficult to perfect. In the case of on-line derivatization methods, the derivatization time must be kept to a minimum. The reaction length with CENU is on average situated between the few seconds necessary for the derivatizations with OPA and the many hours required for the derivatizations with acetylacetone [37]. Shorter reaction time has not particularly been investigated here. It is thus possible to envisage one day the automation of this method on condition that a reaction time of less than 1 min can be obtained. Pre-column derivatization is already easy to implement with the operational conditions described here. This allows one to work with a series of samples, which is not possible with the other methods. The material needs here are minimal.

After having characterized the chromatographic conditions and those for derivatization, the stability of the derivatives was studied. The results show that after reaction the derivative obtained is stable, not reacting to light or temperature change (22 days for the derivatized histamine). This stability arises from the flexibility of handling [36]. The stability of the

derivatives obtained using CENU is much greater than that of the derivatives obtained with OPA (a few hours) [7,10,26], acetylacetone (7 days at ambient temperature without any protection against light) [37], fluorescamine (7 days away from light without any other protection) [19] and aromatic anhydrides (several days at ambient temperature) [24]. The stability of the derivatives is due to the formation of ureas which are known to be very stable compounds [32]. The stability is independent of the derivatized amine.

The derivatives being stable, it is possible to dissociate in time the derivatization phase from that of the chromatographic measurement. The derivatization stage is thus no longer considered as being necessarily linked to the chromatographic injection. The stability of the derivatives also allows one to implement a control at the measurement stage through reinjecting at intervals sample derivatives that have been stored. This method can thus be adapted to all types of laboratory practices and allows implementation of quality control.

The detection limit is directly related to the nature of the chromophore, the column efficiency and the background noise. This method uses CENU agents of derivatization, the chromophore part of which can be modularized. A second chromophore was tested in this study. This chromophore while differing from the previous one through the addition of NO_2 had both the same reaction properties of the derivatizing agent and the stability of the derivative. This modification allowed a reduction in detection limit (DL) by a factor of about 40. This reduction in DL is due to an increase of the ε of the derivative, an increase of the column efficiency and a reduction of the background noise. The detection limit of 0.18 pmol of histamine injected (10 μl) is low. It is difficult to compare different data in this field in view of the large number of ways used in presenting such results.

The ease of modification of the chromophore without changing the reactivity of the molecule allows one to envisage a further reduction in detection limit as well as changing to another detection method (fluorometry).

The modification of the chromophore brings out the possibility of modifying the chromatographic condition as the detection with CENU–$2NO_2$ was slightly less rapid than with CENU–NO_2. The CENUs have a modular structure which allows modifications in both chromatographic conditions and type of detection.

This method has made it possible to derivatize 3 different biogenic amines and detect them on the same chromatogram. The output of derivatization obtained with putrescine is equivalent to that of histamine, while with cadaverine it is 50%. The reaction mechanisms are still badly understood. Such results are probably related to the basicity of the amines and to the derivatization conditions (pH). Depending on the pH conditions it should be possible to obtain a derivatization output of 100% with cadaverine. A deeper study of the different reaction paths is needed.

The specific manner of the reaction of the isocyanates on amines is a further advantage of the derivatization by CENUs. This selectivity allows one to think that within the frame of complex matrices, this method will perform better than those which are less selective. To increase selectivity means to decrease interactions with the matrix, and thus to facilitate the measurement and purifications. We envisage using this type of derivatization agent for the measurement of other molecules carrying primary amine functions.

The reactivity of CENU depends on the pH of derivatization. Therefore it is possible to derivatize amines having different natures in basicity if the pH of the derivatization is adapted. As the stability is independent of the amine, further investigation on catecholamine and amine biological response modifier would be very interesting.

In conclusion CENUs seem to be interesting derivatizing agents owing to their reactivity and their modular structure. The method presented here would seem to offer important possibilities compared to other current methods. In particular, the simplicity of implementation makes it easy to put into wide use with different laboratories producing results which allow comparability. The number of critical parameters is reduced to a minimum which makes the method very robust. Because of the flexibility of the derivatization reagent the field of investigation is wide.

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References

[1] S. Asotra, P.V. Mladenov, R.D. Burke, J. Chromatogr. 408 (1987) 227.
[2] H.M. Zhang, F.-X. Zhou, I.S. Krull, J. Pharmaceut. Biomed. Analysis 10(8) (1992) 577.
[3] K. Saito, M. Horie, N. Nose, K. Nakagomi, H. Nakazawa, J. Chromatogr. 595 (1992) 163.
[4] T.A. Neubecker, N.S. Miller, T.N. Asquith, K.E. Driscoll, J. Chromatogr. 574 (1992) 340.
[5] E. Kasziba, L. Flancbaum, J.C. Fiptzpatrick, J. Schneiderman, H. Fischer, J. Chromatogr. 432 (1988) 315.
[6] S.P. Ashmore, A.H. Thomson, H. Simpson, J. Chromatogr. 496 (1989) 435.
[7] J.P. Gouygou, C. Sinquin, P. Durand, J. Food Sci. 52 (1987) 925.
[8] D. Tsikas, R. Velasquez, C. Toledano, G. Brunner, J. Chromatogr. 614 (1993) 37.
[9] H. Ohta, Y. Takeda, K. Yoza, Y. Nogata, J. Chromatogr. 628 (1993) 199.
[10] G. Turiak, L. Volicer, J. Chromatogr. A 668 (1994) 323.
[11] H. Bruckner, S. Haasman, M. Langer, T. Westhauser, R. Wittner, H. Godel, J. Chromatogr. A 666 (1994) 259.
[12] J.M. Hungerford, K.D. Walker, M.M. Wekell, J.E. Larose, H. Throm, Anal. Chem. 62 (1990) 1971.
[13] V.K. Boppana, C. Miller-Stein, J. Chromatogr. A 676 (1994) 161.
[14] K. Saito, M. Horie, H. Nakazawa, Anal. Chem. 66 (1994) 134.
[15] H. Koning, H. Wolf, K. Venema, J. Korf, J. Chromatogr. 533 (1990) 171.
[16] H. Fukuda, A. Yamatodani, I. Imamura, K. Maeyama, T. Watanabe, H. Wada, J. Chromatogr. 567 (1991) 459.
[17] J. Rosier, C. Van Peteghem, Z. Lebensm. Unsters. Forsch. 186 (1988) 25.
[18] A. Lebiedzinska, H. Lamparczyk, Z. Ganowiak, K.I. Eller, Z. Lebensm. Unsters. Forsch. 192 (1991) 240.
[19] C.M.C.J. Van Haaster, W. Engels, P.J.M.R. Lemmens, G. Hornstra, G.J. Van Der Vusse, J. Chromatogr. 617 (1993) 233.
[20] C.-X. Gao, I.S. Krull, T.M. Trainor, J. Chromatogr. 463 (1989) 192.
[21] T.-Y. Chou, C.-X. Gao, S.T. Colgan, I.S. Krull, C. Dorschel, B. Bidlingmeyer, J. Chromatogr. 454 (1988) 169.
[22] G.C. Yen, C.L. Hsieh, J. Chromatogr. 56 (1991) 158.
[23] P.J. Oefner, S. Wongyai, G. Bonn, Clin. Chim. Acta 205 (1992) 11.
[24] M. Pawlowska, J. Zukowski, D.W. Armstrong, J. Chromatogr. A 666 (1994) 485.
[25] R. Tawa, K. Koshide, S. Hirose, T. Fujimoto, J. Chromatogr. 425 (1988) 143.
[26] S. Smith, T. Sharp, J. Chromatogr. B 652 (1994) 228.
[27] A.M. Uhe, G.R. Collier, E.A. McLennan, D.J. Tucker, K. O'Dea, J. Chromatogr. A 654 (1991) 81.
[28] P. Koski, I.M. Helander, M. Sarvas, M. Vaara, Anal. Biochem. 164 (1987) 261.
[29] P. Furst, L. Pollack, T.A. Graser, H. Godek, P. Stehle, J. Chromatogr. 499 (1990) 557.
[30] R.J. Weinkam, H.-S. Lin, Adv. Pharmacol. Chemother. 19 (1982) 1.
[31] H. Süli-Vargha, J. Bodi, M. Meszaros, K. Medzihradszky, J. Med. Chem. 31 (1988) 1492.
[32] J.H. Yu, G.D. Li, I.S. Krull, S. Cohen, J. Chromatogr. B 658 (1994) 249.
[33] O. Busto, J. Guasch, F. Borrull, J. Chromatogr. A 737 (1996) 205.
[34] M. Pawloska, S. Cohen, D.W. Armstrong, J. Chromatogr. 641 (1993) 257.
[35] J. Martinez, J. Oiry, J.L. Imbach, F. Winternizt, J. Med. Chem. 25 (1982) 178.
[36] K.A. Krok, S.S. Seaver, BioTech. 10 (1991) 664.
[37] Y. Nishikawa, J. Chromatogr. 392 (1987) 349.