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STABILITY OF THE *o*-PHTHALALDEHYDE–HISTAMINE COMPLEX

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SUMMARY

Three methods of derivatization of histamine with *o*-phthalaldehyde (OPT) reagent were compared in order to confirm the stability of the fluorescent product. A reversed-phase high-performance liquid chromatographic system with a fluorescence detector was used. Immediately after derivatization, samples were injected at intervals of 10 min for a total of 60 min. The stability of the fluorescent complex varied according to the method of derivatization. For method 1 (histamine–OPT reaction at ambient temperature) the peak area after 10 min was only half that of its original value. For method 3 (acidification with hydrochloric acid of the end-product of derivatization) only a slight decrease occurred over the 60-min period; method 2 (extraction of the OPT complex with ethyl acetate) was the most stable.

INTRODUCTION

The derivatization of amines and amino acids with *o*-phthalaldehyde (OPT) has been the subject of numerous studies. The chemical reactions involved, however, are not fully understood[1].

In an attempt to quantify histamine in wine using a reversed-phase high-performance liquid chromatographic (HPLC) system, we observed that the histamine–OPT complex prepared according to Subden *et al.*[2] was unstable. A more thorough investigation, using three other methods of derivatization with OPT, was carried out in order to seek confirmation of the instability.

MATERIALS AND METHODS

Two isocratic mobile phases were used, one consisting of methanol–0.08 *M* acetic acid (52:48)[3] and the other acetonitrile–water (40:60) buffered to pH 7.0 with dipotassium hydrogen orthophosphate[2]. The solvents were filtered through a 0.45-μm filter and degassed by sonication just prior to use. A flow-rate of 1 ml/min was used throughout. OPT, mercaptoethanol and histamine were obtained from Sigma

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(St. Louis, MO, U.S.A.). All other chemicals met the ACS specifications. A stock solution of histamine in water (100 mg/l) was prepared, filtered through a 0.45-μm filter and stored at 4°C.

Liquid chromatography

The liquid chromatographic system consisted of a Waters Model 6000A solvent delivery device, a Waters U6K injector and a Waters Model 420 fluorescence detector (Waters Assoc., Milford, MA, U.S.A.). The detector was equipped with an F4T5BL lamp, a 340-nm excitation filter and a 440-nm emission filter; the gain was set at ×64.

A Spectra-Physics Model SP4000 central processor and a Model SP4020 data interface (Spectra-Physics, Santa Clara, CA, U.S.A.) were used for integration and recording.

A μBondapak C_{18} column (Waters Assoc.) was used for reversed-phase HPLC. All analyses were performed in an air-conditioned room at 22°C.

Derivatization

Three methods of derivatization were investigated.

Method 1 (according to Mell et al.[3]. OPT reagent was prepared freshly each day by dissolving 160 mg of OPT in 3 ml of ethanol and 0.2 ml of 2-mercaptoethanol under a ventilated hood. This solution was added to 100 ml of 0.4 mol/l boric acid which was adjusted to pH 9.5 with potassium hydroxide.

For derivatization, 0.1 ml of stock histamine solution was treated with 1 ml of OPT reagent in a Teflon-lined screw-capped test-tube protected against light with aluminium foil and mixed for 1 min on a Vortex mixer. After incubation for 2 min at ambient temperature (20–22°C), 0.9 ml of doubly distilled water was added and mixed. The solution was then ready for injection. The injection volume of 20 μl corresponded to 0.1 μg of histamine. The time required from derivatization to injection was 5 min.

Method 2 (according to Davis et al.[4]). OPT reagent was prepared by dissolving 0.5 g of boric acid in 19 ml of distilled water and titrating with potassium hydroxide solution (450 g/l) to pH 10.40 ± 0.02. After transferring the titrated solution to a dark-glass bottle with a Teflon-lined screw cap, 17.5 mg of OPT were weighed into a 5-ml beaker, dissolved in 200 μl of glass-distilled methanol and added to the borate solution together with 40 μl of 2-mercaptoethanol. The reagent was prepared fresh daily.

For derivatization, an aliquot of 0.5 ml of the histamine stock solution was diluted to approximately 10 ml, the pH was adjusted to 7.0 ± 0.2 with 0.5 mol/l potassium hydroxide solution and the volume was adjusted to 10 ml. A 1-ml volume of this solution was derivatized in a graduated conical centrifuge flask protected against light with aluminium foil by adding 0.5 ml of the OPT reagent. Two grams of sodium chloride were added to break any emulsion formed during two consecutive extractions with 2-ml portions of ethyl acetate. The sample was shaken on a Vortex mixer for 1 min during each extraction followed by sedimentation for 1 min (instead of centrifugation) to separate the phases. After extraction, the ethyl acetate phase was extracted twice with 2-ml portions of dibasic sodium phosphate buffer (50 mol/l, pH 10.0 ± 0.1), shaken for 1 min and allowed to sediment for 1 min. The ethyl acetate

was reduced to 1 ml under oxygen-free, dry nitrogen. The injection volume of 20 μl corresponded to 0.1 μg of histamine. The time required from derivatization to injection was 25 min.

Method 3 (according to Rice et al.[5]). OPT reagent was prepared by dissolving 10 mg of OPT in 1 ml of methanol.

For derivatization, an aliquot of 135 μl of the histamine stock solution was mixed with 1.86 ml of 0.1 *N* hydrochloric acid in a Teflon-lined screw-capped test-tube protected from light with aluminum foil. To this was added 0.4 ml of 1 *N* sodium hydroxide solution and the solution was mixed and left for 1 min. A 0.1-ml volume of the OPT reagent was added and the solution was mixed and left for 4 min, followed by the addition of 0.2 ml of 3 *N* hydrochloric acid. The injection volume of 20 μl corresponded to 0.1 μg of histamine. The time required from derivatization to injection was 7 min.

Procedure

The samples were injected immediately after the derivatization process (time zero), and thereafter at intervals of 10 min for a total of 60 min.

The study was conducted according to a 2 × 3 × 7 split–split plot design in two replications. The factors were, respectively, two mobile phases, three methods of derivatization and seven-fold injection.

RESULTS

The fluorescence of the histamine–OPT complex is represented by the area under the related peak (not shown). Immediately after derivatization (time zero) the

TABLE I

FLUORESCENCE RESPONSES IN TERMS OF OBSERVED AREAS UNDER THE HISTAMINE PEAK AS A FUNCTION OF TIME AFTER DERIVATIZATION (MEAN OF TWO REPLICATES)

Experimental conditions: reversed-phase HPLC system; 2 isocratic phases; 3 methods of derivatization; 7 injections per phase/method/replicate combination; 0.1 μg of histamine injected.

Time after derivatization (min)	*Observed areas under peaks* $\times 10^{-2}$					
	Methanol–acetic acid (52:48)			*Acetonitrile–water (40:60)*		
	Method 1	*Method 2*	*Method 3*	*Method 1*	*Method 2*	*Method 3*
0	1326 (3.12)*	1343	3666	2095 (3.31)*	1461	3434
10	625 (2.80)	1358	3575	1117 (3.04)	1442	3370
20	277 (2.44)	1330	3571	589 (2.76)	1393	3350
30	141 (2.14)	1385	3480	445 (2.64)	1415	3336
40	73 (1.87)	1405	3448	317 (2.50)	1492	3224
50	39 (1.58)	1372	3386	221 (2.34)	1527	3252
60	25 (1.40)	1408	3347	214 (2.33)	1450	3220
Standard error of slope, s_b	50.76 (0.009)	29.32	4.50	68.04 (0.014)	13.27	30.49
Coefficient of determination, r^2	(98.8)	1.1	91.8	(92.0)	3.2	10.3
R^2	73.42			76.1		

* Log_{10} (area).

observed areas for methods 1 and 2 for both mobile phases were about half those for method 3 (Table I).

The fluorescence of the histamine–OPT complex obtained by method 1 decreased markedly during standing; 10 min after derivatization it was half of its original value. Statistical analysis showed that this decrease followed a linear logarithmic curve (r^2 — 98.8 and 92.0 for mobile phases 1 and 2, respectively). Antilogarithms of this linear curve were used to draw the regression lines in Fig. 1 (method 1).

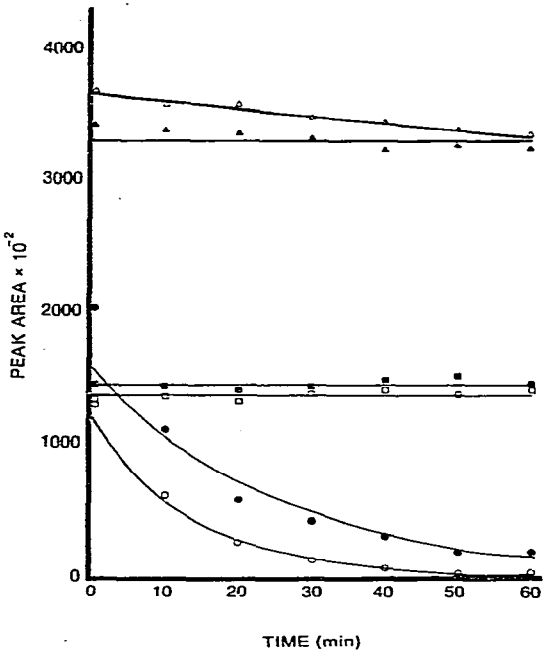

Fig. 1. Regression lines as a function of time of injection on the fluorescence responses of the histamine–OPT complex obtained by three methods of derivatization and using two HPLC isocratic phases (mean of two replicates). Open symbols: methanol–acetic acid. ○, Method 1, $\log_{10} \hat{y}^* = 3.07 - 0.029x$; □, method 2, $\hat{y}^{**} = 1339.2 + 1.07x$; △, method 3, $\hat{y} = 3652.0 - 5.2x$. Closed symbols: acetonitrile–water. ●, Method 1, $\log_{10} \hat{y}^* = 3.20 - 0.016x$; ■, method 2, $\hat{y}^{**} = 1428.9 + 0.84x$; ▲, method 3, $\hat{y}^{**} = 3415.4 - 3.59x$.

Derivatization according to method 2 resulted in a stable complex; any variation in the area was independent of the duration between derivatization and injection (r^2 = 1.1 and 3.2).

A fairly stable omplex was obtained with method 3, although a decrease in fluorescence did occur upon standing when methanol–acetic acid was used as the mobile phase (r^2 = 91.8). With the other mobile phase (r^2 = 10.3), the apparent decrease was not statistically significant.

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* The plotted line was based on the antilog of $\log_{10} \hat{y}$.

** The slopes were not statistically significant, and therefore $\hat{y}$ was taken as $\bar{y}$.

DISCUSSION

Although the choice of a mobile phase is based on optimal resolution, the interrelation that may exist between the solvents and components under investigation should not be neglected. In this work, for two out of the three methods of derivatization methanol–acetic acid gave lower fluorescence values than acetonitrile–water. The acetate anion is reported to affect adversely the fluorescence of the OPT derivatives[1].

Of the three methods used, one had its fluorescence reduced by half 10 min after derivatization, while the other two were fairly stable. Adequate standardization of the procedure (duration of derivatization, interval between derivatization and measurement) is required for reproducibility and accuracy. The sensitivity of a method is also important when small amounts need to be detected and quantified (method 3 *versus* method 1, time zero, or method 2).

CONCLUSION

OPT is a useful reagent for the determination of histamine but careful choice and standardization of a method of derivatization are necessary in order to obtain reproducible data.

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