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Stability of *o*-phthalaldehyde–sulfite derivatives of amino acids and their methyl esters: electrochemical and chromatographic properties

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Abstract

RP-HPLC coupled with 16-channel coulometric electrode array detection was used to monitor the decomposition of five amino acid *o*-phthalaldehyde (OPA)–sulfite derivatives (Ala, Arg, Glu, Ser, Tyr) and their methyl ester derivatives as well. At fixed OPA and sulfite concentrations inclusion of methanol and EDTA in the derivatization media has increased most effectively the room temperature stability of both derivatives measured at pH 9.2 (amino acids) and pH 8.2 (methyl esters). Decreases in product concentrations by 6% have occured after more than 15 h for amino acid derivatives and 8 h for methyl ester derivatives.

The oxidation potential maxima for OPA–sulfite derivatives of amino acids were found at 600 mV while the same methyl ester derivatives had 60–120 mV higher maxima with the exception of tyrosine. The detector responses were found to be linear in the studied 0.1–10 μM concentration range for both derivative forms and their detection limit was 100–200 fmol injected on the column.

The RP-HPLC retention of amino acid methyl ester OPA–sulfite derivatives was very similar to the amino acid OPA–2-mercaptoethanol ones while the more polar amino acid OPA–sulfite derivatives were eluted earlier ($k' < 1$) under the same chromatographic conditions.

1. Introduction

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of a suitable thiol compound to produce fluorescent and electrochemically active 1-alkylthio-2-alkyl-substituted isoindoles [1,2]. This rapid and sensitive derivatization has become widely used in conjunction with HPLC to analyze common amino acids. However the instability of isoindoles limits the utility of the precolumn derivatization method. Careful timing of the reaction or even instrumental automation is required to ensure acceptable analytical precision [3].

Recently various thiol compounds and thiol substitutes have been used to gain more stable primary amine derivatives (reviewed in ref. 4). Among them the well defined sodium sulfite appeared to be a good candidate unlike the volatile and malodorous organic thiols [5]. With the exceptions of few application notes [6,7] no

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detailed and systematic study has been performed yet to characterize the OPA–sulfite derivatives of amino acids.

The investigate OPA-sulfite derivatizations, five amino acids (Ala, Arg, Glu, Ser, Tyr) representing five different amino acid groups and their methyl esters (AlaMe, ArgMe, SerMe, TyrMe and glutamate dimethyl ester, GluDiMe) were selected for this study. Our present objectives have been to calculate the long term stability of OPA–sulfite derivatives of amino acids and their methyl esters and to examine the effect of the organic modifier and EDTA on the concentration decrease. In addition, other properties of the derivatives (electrochemical detectability and RP-HPLC retention) which could affect the general liquid chromatographic utility of these products were also investigated.

2. Experimental

2.1. Chemicals

OPA, 2-mercaptoethanol (2-ME), amino acid and methyl ester standards were purchased from Sigma (St. Louis, MO, USA), sodium sulfite, buffer constituents, HPLC-grade water and methanol were from Fisher Scientific (Fair Lawn, NJ, USA) All chemicals were used as received.

2.2. HPLC procedure

Gradient HPLC system equipped with autosampler and IBM Model 286 computer was used in this study (Neurochemical Analyzer, ESA, Bedford, MA, USA). The coulometric electrode array consisted of 16 porous graphite electrodes in series, the oxidation potentials were set from 0 to 960 mV in 60-mV increments. The analytical column used was an Ultracarb (20) ODS, 5 μm, 150 × 4.6 mm (Phenomenex, Torrance, CA, USA) and its temperature was set 35°C. Mobile phase A consisted of 29 m*M* phosphate buffer (pH 7.1) and 0.1 m*M* EDTA while mobile phase B had 65 parts A and 35 parts methanol. The separations were run at 1 ml/min flow-rates, mobile phase B was used to resolve the amino acid methyl ester OPA–sulfite derivatives and to compare the retention and electrochemical properties of all derivatives. Linear gradient (0 min: 8% B; 4 min: 8% B; 22 min: 70%B) was run to separate the amino acid OPA–sulfite derivatives. Hold-up time for k' calculations was determined by injection of 0.05 *M* borate buffer.

2.3. Derivatization procedure

The OPA–2-ME derivatizations were performed as described earlier [8]. OPA (0.15 mg/ml) and 0.1 mg/ml sodium sulfite in 0.05 *M* borate buffer (pH 9.2 for amino acids and pH 8.2 for methyl esters) were used for sulfite derivatizations, and 20% (v/v) methanol and/or 4 μg/ml EDTA was used for stability studies. Typically 10 μl of standard mixtures (individual amino acid and methyl ester concentrations of approximately 0.15 mg/ml) were combined with 990 μl derivatization mixtures and were vortexed for 30 s. The amber glass vials were kept at 20°C on the autosampler rack and 10-μl aliquots were injected onto the HPLC column at different time intervals.

3. Results

Our first experiments studied the RP-HPLC retention and separation of the OPA–sulfite derivatives of the model compounds. As a starting point we selected a mobile phase solvent strength which is used to separate the amino acid OPA–2-ME derivatives [8] and we have used them for the comparisons of these derivatives as well. The underivatized, electrochemically active tyrosine and tyrosine methyl ester were also investigated and the results are summarized in Table 1.

As we expected the amino acid OPA–2-ME derivatives were eluted in the 0.7–8.49 capacity factor range, while the same sulfite derivatives did not retain at all with the k' values less than 0.68. The methyl ester OPA–2-ME derivatives proved to be very non-polar and had high k' values while the OPA–sulfite derivatives had a

Table 1
k' values for native and derivatized (OPA–2-ME, OPA–sulfite) amino acids and methyl esters

	Amino acids			Methyl esters		
	Native	Derivatives		Native	Derivatives	
		2-ME	Sulfite		2-ME	Sulfite
Alanine		8.08	0.37		69.31	2.97
Arginine		4.03	0.44		27.81	2.09
Glutamate		0.70	0.17		94.84[a]	5.72[a]
Serine		2.12	0.27		18.38	1.09
Tyrosine	0.32	8.49	0.68	1.97	>115	11.90

See Experimental section for HPLC conditions.
[a] Dimethyl ethers.

reasonable retention at this solvent strength between 1.09 and 11.9. This capacity factor range was about the same for the amino acid OPA–2-ME derivatives. In general the OPA–sulfite derivatives were essentially more polar than the OPA–2-ME ones and the methyl ester derivatives were retained longer on RP-HPLC than the corresponding amino acid ones. In certain cases the relative retention order was also affected by the different derivatizations or esterification (Table 1). Besides the model compounds other amino acids were also detected with the OPA-sulfite derivatizations. We could observe single, uniform peaks for Asn, Asp, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Tau, Thr, Trp, Val, γ-amino-*n*-butyric acid as well as for cysteic acid and dipeptides carnosine and homocarnosine. Most of these amino acid OPA–sulfite derivatives were identified from the 0.1 *M* perchloric acid extracts of different monkey brain tissues as well.

Parallel with the retention studies the electrochemical behaviour of the same derivatives was also investigated (Table 2). Each derivative had a characteristic electrochemical pattern, a dominant oxidation potential where the strongest signal occured and other subdominant potentials with weaker signals. The peak height ratios of the different potentials were highly reproducible if the mobile phase composition was kept constant. The peak heights of the dominant electrodes were considered 100% and the other subdominant signals were expressed as a fraction of it. Ala, Arg, Ser had the same electrochemical behaviour. Their OPA–2-ME derivatives were oxidized at the lowest potentials, while their methyl ester derivatives had 60–120 mV higher oxidation potential maxima. The sulfite derivatives of these amino acids were oxidized at 180 mV higher than the corresponding 2-ME ones, in case of methyl esters this difference has increased by 60 mV. These results indicate that esterification resulted in a slight increase in the oxidation potential maxima for the same derivatives, while the products of the sulfite derivatizations were more difficult to oxidize than the corresponding 2-ME ones. The Glu OPA–2-ME derivative had higher oxidation maximum than expected while the inherent electrochemical activity of Tyr and TyrMe was affected less by the different derivatizations.

Because of the lack of any information on the pH dependent formation of sulfite derivatization of amino acids and their methyl esters we also investigated the suitable derivatization pH range (Fig. 1). Standard mixtures were derivatized at different pH in phosphate and borate buffers and the maximum electrochemical responses were determined by repeated injections at different time intervals from the beginning of derivatization. In the case of amino acids the electrochemical signals of the sulfite derivatives were increased at alkaline pH, and were approximately the same between pH 8 and 10. The increased

Table 2
Comparative electrochemistry of native and derivatized (OPA–2-ME, OPA–sulfite) amino acids and their methyl esters

		Electrode array oxidation potentials in mV							
		300	360	420	480	540	600	660	720
Alanine	2-ME		41.3	*100*	27.0				
	Sulfite					43.7	*100*	29.4	
Methyl ester	2-ME			76.9	*100*	68.1			
	Sulfite						35.2	*100*	88.5
Arginine	2-ME	71.3	*100*	34.4					
	Sulfite			26.2	20.3	93.2	*100*	12.0	
Methyl ester	2-ME			29.6	*100*	42.2			
	Sulfite						87.2	*100*	31.7
Glutamate	2-ME		25.0	78.0	46.8	*100*	66.9		
	Sulfite					34.8	*100*	69.1	
Dimethyl ester	2-ME				53.7	*100*	13.0		
	Sulfite						21.4	86.3	*100*
Serine	2-ME		43.2	*100*	40.3	39.8			
	Sulfite					47.2	*100*	61.5	
Methyl ester	2-ME			83.3	*100*	80.6			
	Sulfite						57.3	*100*	29.3
Tyrosine	Native					20.6	*100*	64.2	
	2-ME		36.2	*100*	44.6	61.7	69.0	47.4	
	Sulfite					61.5	*100*	37.5	
Methyl ester	Native					33.3	*100*	33.1	
	2-ME				68.0	*100*	68.0		
	Sulfite					30.2	*100*	28.1	

The specific oxidation potentials are presented, where the peaks appeared on the electrode array. *100* represents the maximum peak height at the leading channel; peak heights at subdominant channels are expressed as a percentage of the peak height. See Experimental section for HPLC conditions.

isoindole formation in alkaline media is in good agreement with earlier data obtained with organic thiols [1] but we have not experienced considerable differences between the phosphate and borate buffers at the same pH value. The pH-dependent plot for the amino acid methyl ester sulfite derivatives shows a different shape. In the neutral pH range the derivative formation was not complete while the strongly alkaline media saponified the methyl ester groups. The formation of serine methyl ester at pH 8.2 in phosphate buffer was less than in borate buffer and it was best formed under weak acidic condition. On the basis of these results, we decided to derivatize the amino acids at pH 9.2 and the methyl esters at pH 8.2 both in borate buffer.

Before the stability studies the linearity of the electrochemical responses was also investigated. Five different dilutions of amino acid or methyl ester standard mixtures were derivatized the same way and the average peak area values of duplicate determinations were plotted against the concentrations. Linear correlation coefficients of at least 0.999 were obtained for each derivative in the investigated 0.1–10 μM concentration range. Further dilutions were made to establish the detection limit. Although at this point of our research the separations were not optimized, we could identify the sulfite derivatives in the 100–200 fmol range ($S/N = 2$). The same experiments were performed with the amino acid OPA–2-ME derivatizations as well and similar linear correlation coefficients (>0.999) and range of detection limits were established.

The long term stability of amino acid and methyl ester OPA–sulfite derivatives was determined by monitoring their decomposition for a 23-h period. In Fig. 2 arginine and arginine methyl ester serve as a typical example. Usually the first samples, 1 h following the derivatization were considered 100% and the later injections

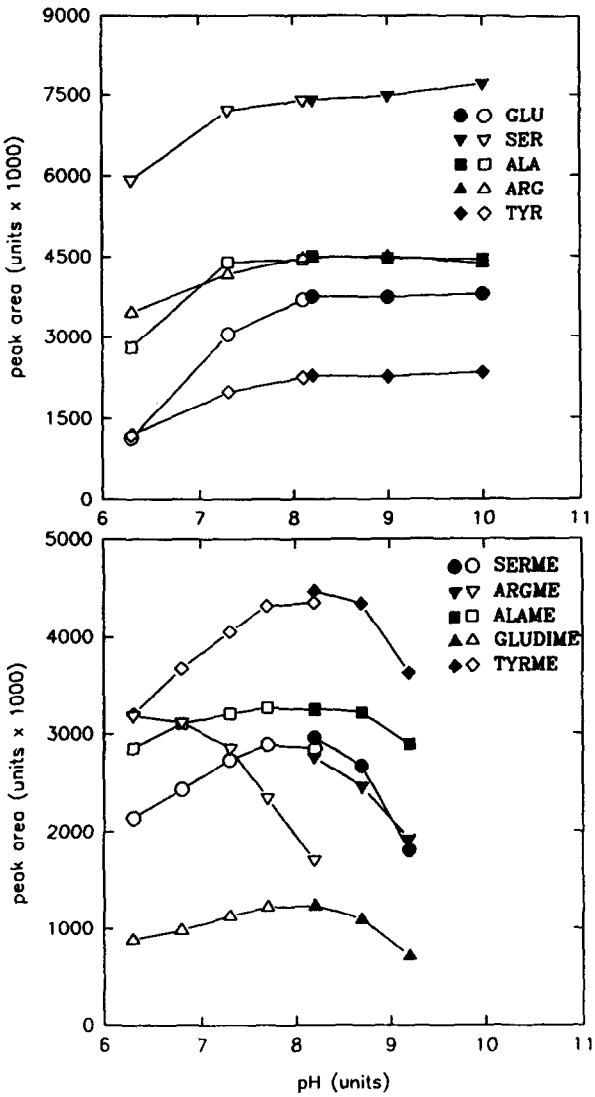

Fig. 1. pH-dependent formation of OPA–sulfite derivatives of amino acids and their methyl esters in phosphate (opened labels) and borate (filled labels) buffers. Each point represents the average of three measurements.

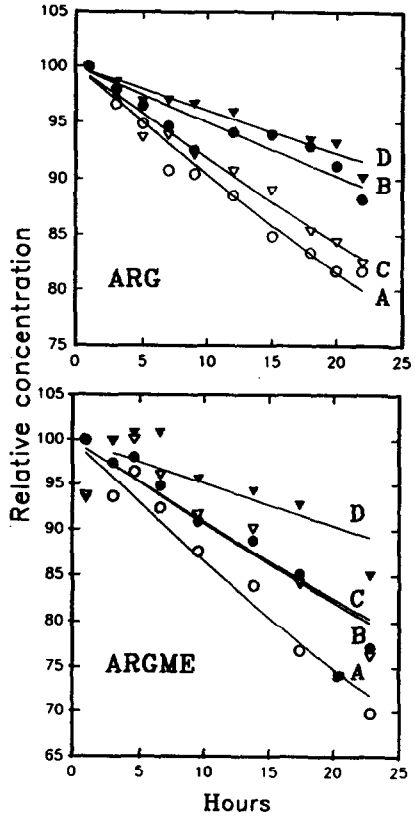

Fig. 2. Long term degradation of OPA–sulfite derivative of arginine and arginine methyl ester (see Table 3 for differences in A, B, C and D derivatizations).

were related to these values. The derivative formation of ArgMe, GluDiMe, SerMe and TyrMe was not complete in 1 h if 20% methanol was involved and in these cases the second time (3 h) point was considered 100%. Earlier observations on the decomposition of primary amine isoindole sulfonates [5] suggested a pseudo-first-order kinetics, and we have calculated and plotted the ideal lines according to this relationship.

To characterize and compare the stability of each derivative we decided to calculate the times required for 6% decrease ($t_{6\%}$) in product concentration (Table 3). Even in the aqueous, buffered derivatization media the amino acid OPA–sulfite derivatives exhibited good stability at room temperature and the $t_{6\%}$ values were several hours. Involvement of EDTA or 20% methanol in the derivatization media has increased the stability. Tyrosine was less affected by these changes while glutamate showed extremely good stability. When both EDTA and methanol were involved, more than 15 h were needed even for the tyrosine concentration to decrease by 6%. We have also calculated the theoretical X values from the A, B and C

Table 3
Long-term stability of OPA–sulfite derivatives of amino acids and their methyl esters in four derivatization mixture

	D. mix.	Amino acids			Methyl esters		
		n	$t_{6\%}$	S.D.	*n*	$t_{6\%}$	S.D.
Alanine	A	10	9.25	0.56	8	11.01	0.57
	B	10	14.39	1.35	8	15.10	0.58
	C	10	12.14	0.53	8	16.29	0.95
	D	10	21.34	1.01	8	>25	
	X		17.28			20.38	
Arginine	A	10	6.09	0.19	8	4.26	0.22
	B	10	12.07	0.94	8	6.28	0.37
	C	10	7.24	0.21	7	6.43	0.71
	D	10	15.44	0.72	7	12.26	2.20
	X		13.22			8.45	
Glutamate	A	10	23.24	2.50	8	11.01	0.50
	B	10	>25		8	>25	
	C	10	>25		7	>25	
	D	10	>25		7	>25	
	X						
Serine	A	10	9.97	0.69	8	2.26	0.09
	B	10	11.84	0.97	8	2.83	0.16
	C	10	9.42	0.49	7	7.60	0.51
	D	10	16.33	0.99	7	8.74	0.66
	X		11.29			8.17	
Tyrosine	A	10	12.58	1.02	8	17.99	1.01
	B	10	13.14	1.05	8	>25	
	C	10	12.63	0.91	7	24.37	1.80
	D	10	15.55	0.95	7	>25	
	X		13.19				

$t_{6\%}$ = 6% Drop in the initial product concentration (h); *n* = number of observations between 1 and 23 h; SD = standard deviation; D. mix. = derivatization mixture; A = aqueous; B = 0.01 m*M* EDTA included; C = 20% (v/v) methanol; D = both EDTA and methanol included; *X* = calculated theoretical value (B − A + C).

experiments. First we subtracted the $t_{6\%}$ values of A experiments (derivatizations performed in aqueous media) from the B ones (derivatizations in the presence of EDTA) with the difference representing the contribution of EDTA on stability increase. These values were added to the $t_{6\%}$ values of C experiments (20% methanol in the derivatization mixture) representing the theoretical addition of EDTA and methanol effect on the stability. These theoretical values were always less than the measured ones (D experiments, both EDTA and methanol in the derivatization mixture) suggesting an enhancement effect between the EDTA and methanol. The same results were found with the methyl ester derivatives. Compared to the amino acids, TyrMe was more stable than Tyr unlike SerMe which had poorer stability than Ser. The other amino acid and methyl ester pairs had approximately the same stability range.

4. Discussion

Our results indicate that very stable amino acid and methyl ester OPA–sulfite derivatives are formed in the presence of 0.01 m*M* EDTA and 20% methanol. Earlier the formation of 1-isoindole sulfonate structure was proposed for this derivatization using isopropylamine as reactant [5].

Expensive and complex instrumentation is not

needed for the derivatizations because the derivatives are stable at room temperature. The measured stability range is competitive or even better than the recently introduced isoindole derivatizations when naphthalene-2,3-dicarboxaldehyde and cyanide [9] or OPA and N-acetyl-L-cysteine were used [10].

Earlier experiments suggested stability enhancement effect of organic solvents [11,12] on the OPA–organic thiol derived isoindoles and our results showed the same effect on the sulfite ones as well. However, care should be taken to choose the proper organic solvent concentrations because high proportions or anhydrous conditions can slow down the rate of derivative formation.

No reports were found on the influence of EDTA or other metal chelator on the isoindole stability. It is possible that EDTA acts by extracting metals because its concentration was more than one magnitude less than that of the other reactants. The autoxidation process which is thought to be responsible to some extent for the isoindole degradation [13,14] is dependent on the amount of contaminating metal ions in the reagents [15]. The extraction of trace metals would slow down the autoxidation process and improve the overall stability.

The OPA–sulfite derivatives of amino acids and their methyl esters, similar to other isoindoles, are good subjects for electrochemical detections and approximately 180 mV increase in the oxidation potential maxima was established compared to the traditional 2-ME derivatives. The same shift was earlier observed when primary amines were used instead of amino acids [5]. The coulometric electrode array proved to be sensitive and accurate for the identification and the quantification of these compounds.

The pH-dependent formation of OPA–sulfite derivatives of amino acids showed a maximum plateau above pH 8, which was independent from the type of buffers. The same methyl ester derivatives had a maximum at pH 8 with the exception of serine methyl ester which was best formed under weak acidic conditions.

Interestingly the amino acid methyl ester OPA–sulfite derivatives had the same retention range on the RP-HPLC column as the amino acid OPA–2-ME ones. We could identify the neurotransmitter amino acid OPA–sulfite derivatives from the perchloric acid extracts of monkey brain tissues as well. Our latest efforts to separate the full amino acid spectrum suggest that the highly polar amino acid OPA–sulfite derivatives can be separated after complex optimization procedures at weak acidic conditions in the presence of ion pairing reagents and these results will be published later.

5. Acknowledgements

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6. References

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