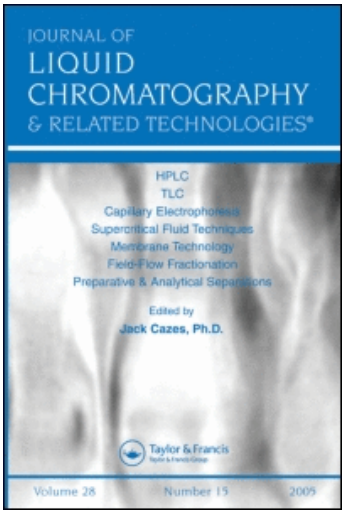

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Derivatizing Reagents Based on Ferrocene for HPLC-Ecd Determination of Peptides and Proteins

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DERIVATIZING REAGENTS BASED ON FERROCENE FOR HPLC-ECD DETERMINATION OF PEPTIDES AND PROTEINS

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ABSTRACT

Several ferrocenic compounds for derivatization of peptides and proteins were synthesized and then tested by reaction with bovine serum albumin (BSA). The reactivity of the reagents and the electroactivity of the derivatized BSA were estimated by the height of the ECD-signal after an HPLC analysis run. The most suitable reagent was 3-ferrocenylpropionic anhydride which reacts with BSA within 15 minutes at room temperature. The anhydride presented itself as a stable compound which can be synthesized in high yields. Up to pH 9 - 10 it is only slowly hydrolyzed, and its derivatization products are highly electroactive. Another reagent is especially suited to derivatize those proteins whose isoelectric points are higher than 10: Ferrocenylmethyl-succinimidyl-glycine-hydrochloride. This compound develops its derivatization activity only with pH values higher than 9, but it is rather difficult to prepare.

INTRODUCTION

Determination of peptides and proteins of low concentration in biological fluids after an HPLC

analysis run affords a highly sensitive detection method. One of the most sensitive detectors for HPLC is the electrochemical detector (ECD) which has been developed in the past two decades (1).

Only a few amino acids show a little electroactivity: Tyrosine (2), tryptophan (3), and methionine (4), for example. To determine those amino acids or peptides containing at least one of those amino acids a rather high oxidation potential (> +850 mV) is necessary (2 - 7). But, high oxidation potentials are not useful because of the decrease in selectivity.

Therefore, an electrophoric group has to be introduced to peptides and proteins. We decided for the ferrocenic group as the electrophore because the ferrocene is easily oxidized to the ferricinium kation (8,9) and that's why an oxidation potential of +400 to +500 mV is enough. Thus, derivatization of amines (10), steroids (11), fatty acids (12), and amino acids (13) with ferrocene allows their sensitive and selective detection after an HPLC analysis run.

Ferrocene's electroactivity is even higher than that of the catechol residue whose oxidation to the o-quinone at +600 mV used for the determination of plasma catecholamines and their metabolites in the picogram range is one of the best known examples for the routine use of HPLC-ECD (14 - 17).

Furthermore, the ferrocene residue is stable enough for the preparation of activated acids which are

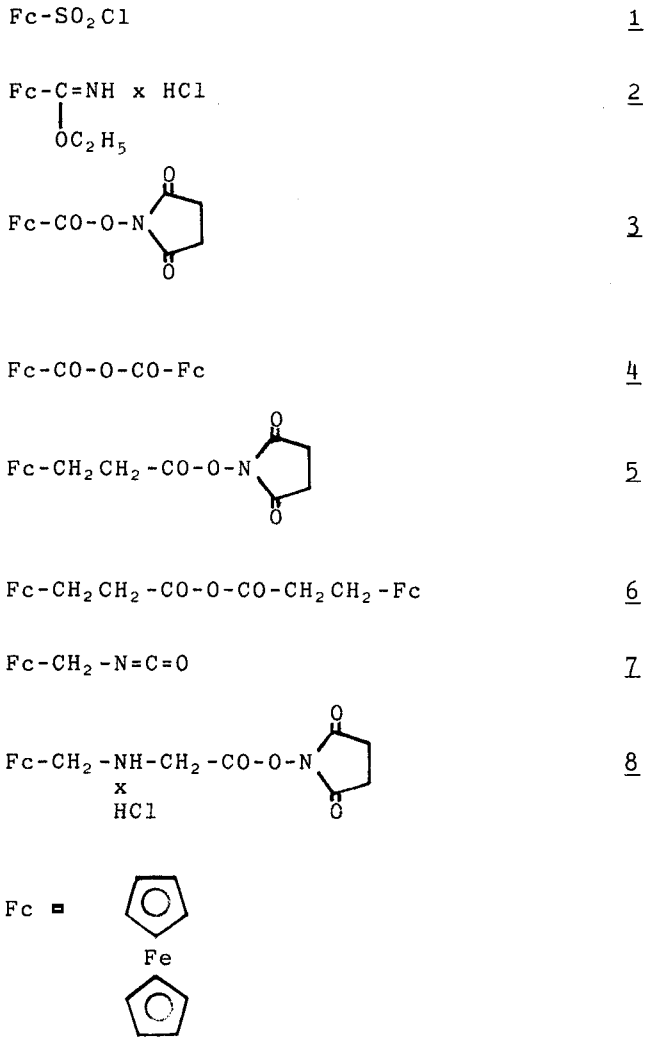

FIGURE 1: Formulas of ferrocene derivatizing reagents

then reacted with the amino groups of the peptides and proteins. Another advantage of the ferrocene group is its bright color which allows easy purification of the ferrocenic compounds by column chromatography.

We synthesized several ferrocenic compounds and tested them first by reaction with phenylalanine-tert-butylester in order to characterize the products and to roughly estimate their reactivity. The syntheses and the results of the test reactions with H-Phe-O-tert-Bu are reported elsewhere (18,19).

MATERIALS AND METHODS

HPLC apparatus. The HPLC system consisted of a Model 510 pump, a Models 460 electrochemical detector with a potential set at +500 mV vs. Ag/AgCl, an automated sample processor WISP 712, and a Protein-Pak I-125 column (all from Waters Ass., Milford, MA). The data were collected by a Waters Model 860 (MicroVax).

Reagents. The ferrocenic compounds were all synthesized (18). The proteins were all purchased from Sigma Chemie (München, F.R.G.). The salts used in the mobile Phase were "p.A."-grade (Merck, Darmstadt, F.R.G), and the acetonitrile was "HPLC"-grade (Promochem, Wesel, F.R.G.). The water was prepared by a Milli-Q-apparatus (Millipore GmbH, Eschborn, F.R.G.).

Mobile Phase. 13.2 g ammonium sulfate (100 mmol) and 0.75 g tartaric acid (5 mmol) were diluted to 1000 ml with Milli-Q-water. To 600 ml of this solution 400

ml acetonitrile were added. The mixture was then degassed in an ultrasonic bath. (Flow rate: 1 ml /min)

Buffer solutions. pH 6.8: 10.4 g K_2HPO_4 and 7 g KH_2PO_4 (together 1.2 mol) were diluted to 100 ml with water. pH 9.1: 1.55 g Na_2HPO_4 x 2 H_2O (0.1 mol) were diluted to 100 ml with water. pH 12.3: 3.8 g Na_3PO_4 x 12 H_2O (0.1 mol) were diluted to 100 ml with water.

BSA solutions. 500 mg of BSA were diluted to 50 ml with water (c = 10 mg/ml). From this solution we took 10 ml and diluted them to 100 ml (c = 1 mg/ml). Again, we took 10 ml and diluted them for another time to 100 ml with water (c = 0.1 mg/ml).

Solutions of the derivatizing reagents. 25 mg of each reagent 1 - 8 (formulas see figure 1) were diluted to 25 ml with acetonitrile except 1 and 2, which were diluted with ethylenglycolmonomethylether and water, respectively (c = 1 mg/ml).

Sample preparation. 50 - 500 l protein solution + 100 l buffer solution + 100 l solution of derivatizing reagent (total volume = 700 l for each sample) were mixed and incubated at room temperature, +40 C, +50 C or +55 C for at least 15 minutes. Then, 10 l of the mixture were directly injected into the HPLC-system.

Data acquisition. For the evaluation of the data we directly used the peak areas given by the ECD signals. No underivatized proteins can be detected under conditions described here.

TABLE 1

Reaction Conditions for Reagent 1 - 8 with BSA:

Reagent	pH	temperature	incubation time
1	12.3	+50 C	2 h
2	9.1	+55 C	20 h
3	6.8	+40 C	1 h
4	6.8	RT	30 min
5	6.8	+40 C	1 h
6	6.8	RT	15 min
7	6.8	RT	30 min
8	9.1	RT	30 min

RESULTS AND DISCUSSION

The following reagents were tested by reaction with BSA:

At first, we determined the reaction conditions for reagents 1 - 8 (listed in table 1).

In spite of the high temperatures and the long incubation times for 1 and 2 the ECD signals were only very poor, even at higher potentials. Thus, these two reagents are not useful at all for a routine method. (We found the same poor reactivity in the reaction with H-Phe-O-tert-Bu (18).)

To estimate the electroactivity of the different BSA derivatization products we measured the current [nA] (ECD signal) at the best potential (evident in the hydrodynamic voltamograms) of each product and set it into relation to the concentration of the concerning

TABLE 2

Reactivity and Electroactivity Factor:

Reagent	Potential [mV]	Current [nA]	Reagent amount [nmol]	Factor [nA/nmol]
3	950	0.410	305	0.0013
4	950	1.737	226	0.0077
5	500	5.568	281	0.0198
6	500	13.997	420	0.0333
7	500	10.739	664	0.0162
8	500	11.056	246	0.0449

reagent. The higher the factor [nA/nmol] the higher the reactivity of the reagent *and* the higher the electroactivty of the product. The BSA concentration was kept constant for this experiment. The results are summarized in table 2.

The first evident result is that the ferrocene carbonic acid derivatives 3 and 4 produce BSA derivatives of very low electroactivity (potential: +950 mV). This is a potential range with significantly low selectivity. Thus, 3 and 4 won't be the reagents of choice for a successfull derivatization method.

Among reagents 3 - 8 are two pairs of compounds which each introduce the same group into the protein and therefore the products have the same electroactivity: 3 and 4 as well as 5 and 6. The different values of the factors concerning the pairs show that

the anhydrides 4 and 6 are more reactive than the succinimidyl esters 3 and 5.

On the other hand, comparison of the factor values for the anhydrides 4 and 6 and those of the succinimidyl esters 3, 5 and 8 shows that the factor is the higher the more spacers are between the ferrocenyl residue and the reaction center.

Shimada et al. (11) who observed the same effect derivatizing the hydroxyl groups of steroids with FcCOCl or FcCOCN (+800 mV, low reactivity), and $Fc(CH_2)_2COCl$ (+360 mV, higher reactivity), respectively interpret these results with the electron-withdrawing effect of the carbonyl group if this is next to the ferrocene group. In other words, the two methylen groups facilitate the oxidation of the ferrocenic group by stabilizing the ferricinium kation with their +I-effect.

We think, besides these electronic effects there must be a simple kinetic effect, too. If the distance between the voluminous ferrocene group and the protein is too small, the reaction itself as well as the oxidation of the ferrocenic group is sterically hindered. If the distance is prolonged the rotational barriers are lowered and the ferrocenic group can take a position of less strain. This means higher reactivity for the reaction with the protein and higher oxidation rates at the working electrode of the ECD.

This kinetic effect might explain why Shimada et al. (11) successfully derivatize the steroids with $FcCON_3$ which forms an urethan with the OH-groups (Fc-NH-CO-R). This urethan without the +I-effect of methylen groups carries one spacer and can easily be oxidized at +400 mV.

Our reagent 8 carries two methylen groups, but they are separated by a protonated amino group. Thus, their +I-vectors are not of a unique direction. Despite of this, 8 has the highest factor and is the only reagent with three spacers in table 2.

With reagent 5 - 8 we performed another test to estimate their reactivty and their sensitivity for hydrolyzation. The concentration of each ferrocenic reagent was kept constant, whereas the BSA concentration was varied. Figures 2 - 5 represent the relation of BSA concentration vs. peak area for each derivatizing reagent. The dotted lines mark the point where saturation of BSA at the given reagent concentration is reached.

The relation between reagent amount and saturation point gives a factor which we called the "surplus factor". The higher this factor the more ferrocenic groups can be introduced into the BSA molecule by the concerning reagent and the higher its reactivity. Table 3 summarizes the results.

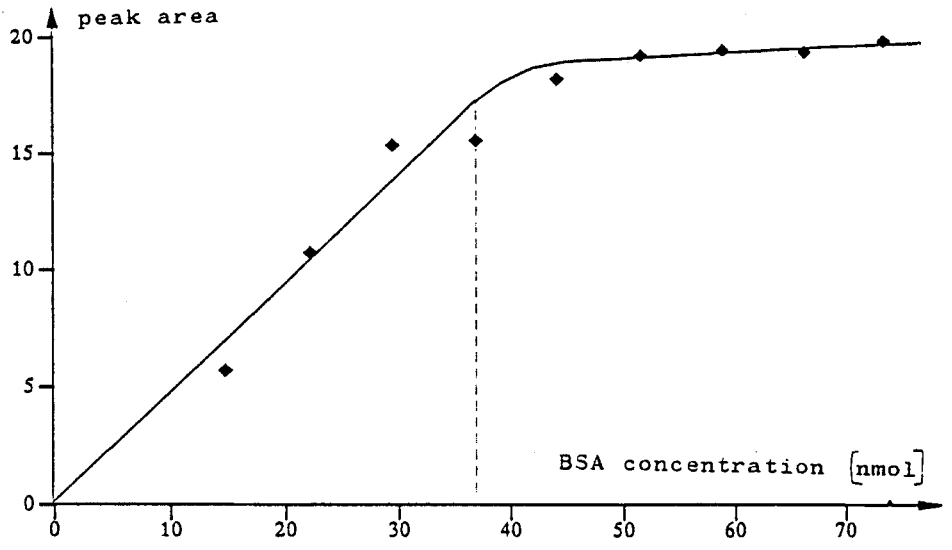

FIGURE 2: Reaction of 3-ferrocenyl-succinimidylpropionat (5)

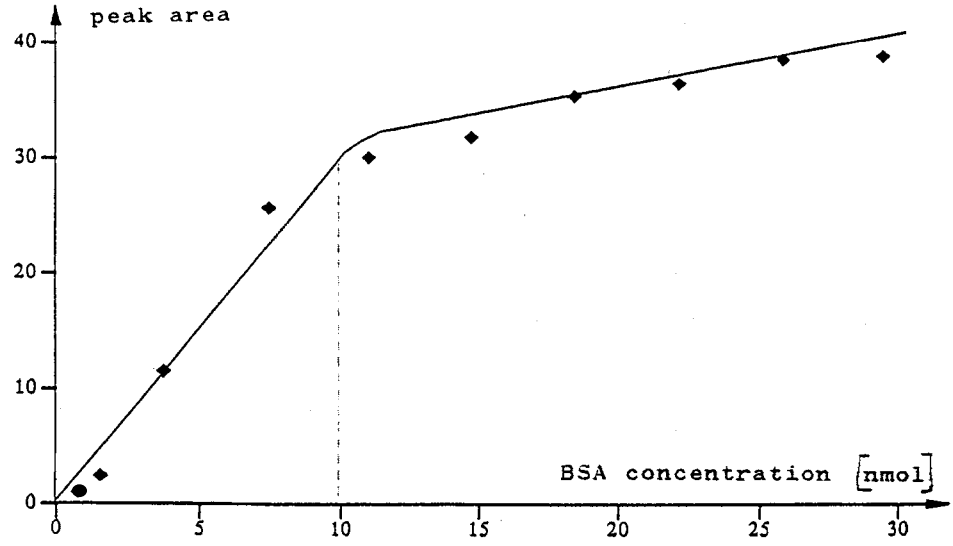

FIGURE 3: Reaction of 3-ferrocenylpropionic anhydride (6)

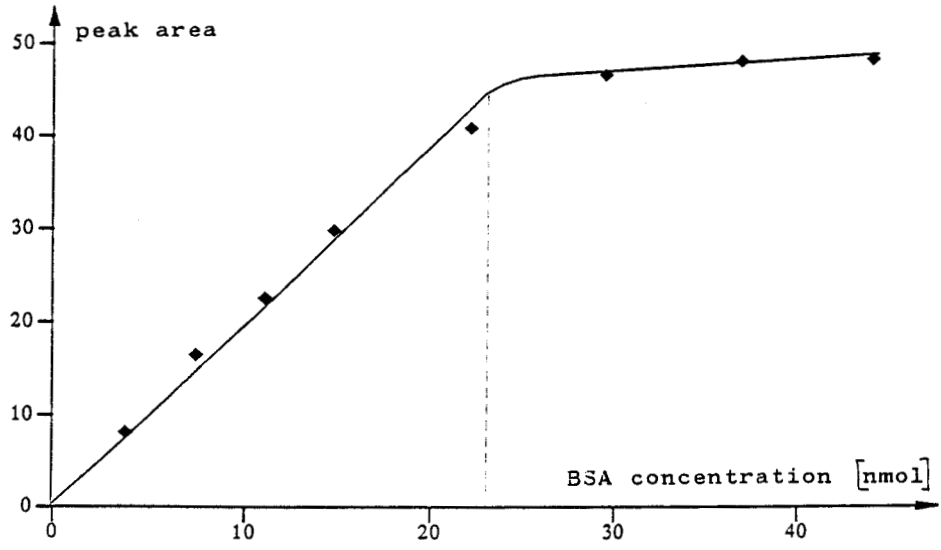

FIGURE 4: Reaction of ferrocenylmethylisocyanat (7)

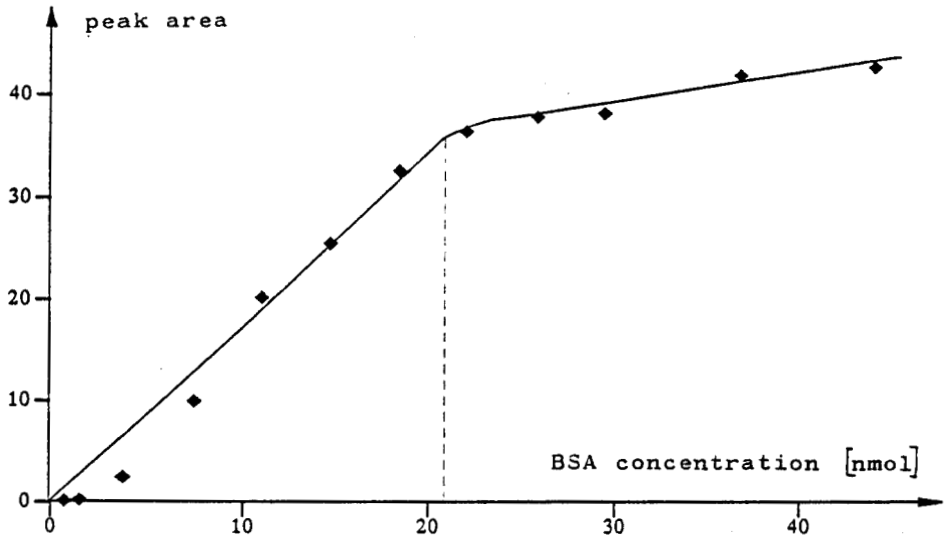

FIGURE 5: Reaction of (ferrocenylmethyl)-succinimidyl-glycine-hydrochloride (8)

TABLE 3

Reactivity Estimation by Determination of the "Surplus Factor":

Reagent	Reagent amount [nmol]	BSA amount at saturation point [nmol]	Surplus Factor [nmol/nmol]
5	280	37	7.6
6	422	10	42.2
7	606	23	26.3
8	542	21	25.8

This means, reagent 6 (3-ferrocenylpropionic anhydride) is the most reactive one and is least hydrolyzed of all.

It is obvious that the reagent solution is not stable over a longer period. To receive reproducible results the solution has to be renewed at least after one week. But the precision within a day is very good: All coefficients of variation are below 5 %.

Furthermore, we tested reagent 6 with a few different proteins: Chymotrypsinogen (c = 0.95 mg/ml in water), ß-lactoglobuline (c = 1.01 mg/ml, in water), -caseine (c = 1.03 mg/ml in water), lysozyme (c = 1.38 mg/ml in water), hemoglobine (c = 1.69 mg/ml in water), cyctochrome C (c = 0.68 mg/ml in water), ribonuclease (c = 0.36 mg/ml in water), fibrinogen (c = 1 mg/ml in water), myoglobine (c = 1 mg/ml in water), and ovalbumine (c = 1 mg/ml in water).

TABLE 4

Precision of the Derivatization of BSA with Reagent 6:

Series	Peak Area mean value (n = 10)	Standard deviation	Coefficient of variation [%]
1	26.3	1.23	4.68
2	24.1	0.91	3.78
3	24.3	0.95	3.91
4	24.8	0.98	3.95
5	22.5	0.91	4.04
6	24.8	0.56	2.26
7	21.0	0.60	2.86
8	15.3	0.68	4.44
9	15.6	0.69	4.42
10	16.3	0.65	3.99

We found the following relations between protein and grade of derivatization:

1. The higher the isoelectric point of the protein the higher the pH value of the reaction medium has to be in order to increase the number of the reaction centers (lysine side chains, for example).
2. The higher the pH value of the reaction medium the higher the hydrolization rate of reagent 6 which is evident in the significant decrease of the ECD signal at higher pH values if the isoelectric point of the protein is below 7. (For proteins with isoelectric points > 7 the increase of reaction centers at higher pH values covers the loss of reagent activity.) This means, proteins

like lysozyme (which has an isoelectric point of 11) are better derivatized with reagent 8 whose derivatizing activity starts at pH values higher than 9 (see table 1) because of its salt structure.

3. Comparing two proteins which have about the same isoelectric point the grade of derivatization (height of the ECD signal) is the higher the higher their number of basic amino acids (relative and absolute). The absolute number of basic amino acids depends on the molecular weight of the protein. This means, the grade of derivatization is the higher the higher the molecular weight of the protein is. Therefore, the detection limit which depends on the grade of derivatization is different from protein to protein. Generally, the detection limit is found in the low picomole range.

ACKNOWLEDGEMENT

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