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COMMUNICATIONS

Micellar Catalysis of an Analytical Reaction: Spectrophotometric Determination of Amino Acids and Peptides after Cetrimonium Bromide-Catalyzed Reaction with 1-Fluoro-2,4-dinitrobenzene

Keyphrases □ Surfactants—cetrimonium bromide, micelle formation, catalysis of amino acid and peptide derivatization, spectrophotometry of derivatives □ Amino acids—spectrophotometric determination following derivatization, catalysis by surfactant micelles □ Peptides—spectrophotometric determination following derivatization, catalysis by surfactant micelles

To the Editor:

Surfactants can alter chemical reaction rates through their micelle-forming capabilities. During the past decade, intensive study of this phenomenon has been stimulated by the hope that micellar catalysis of organic reactions might be a useful model of enzyme catalysis. Although the dynamic and fluid structure of micelles limits their utility as enzyme models, micelle-induced alterations in reaction rates are worth studying on their own account (1, 2). It was suggested (3, 4) that micellar catalysis might be exploited for organic analytical purposes, but no such experimental studies have been reported. We describe the use of micellar catalysis to increase the derivative formation rate prior to spectrophotometric measurement of a product.

1-Fluoro-2,4-dinitrobenzene undergoes aromatic nucleophilic substitution by amines to give arylated amines. This reaction was employed to study the amino acid sequence of insulin (5), and it has since been adapted to the analysis of other amines and polyamines (6–8). The reaction is carried out in an alkaline medium, and the product is measured spectrophotometrically. For the determination of many amines and amino acids, the reaction was essentially complete (within 5% of its maximum) for most samples in 10 min at 65°; some compounds required ≥20 min at this temperature (8).

Several surfactants, notably the cation cetrimonium bromide (cetyltrimethylammonium bromide), catalyze substitution reactions between fluorodinitrobenzene and amine nucleophiles (9); rate enhancements of ~five- to 30-fold were found. Therefore, this reaction appeared to provide an appropriate system for testing the proposal (3, 4) that micellar catalysis may be analytically useful.

Rates were measured for the substitution reaction between fluorodinitrobenzene and some amino compounds in the presence and absence of cetrimonium bromide. In these reactions, the fluorodinitrobenzene was in excess, as it would be in an analytical system; samples were withdrawn and examined spectrophotometrically by a literature method (8). The kinetics were apparent first order over at least two half-lives. Table I gives the half-lives and conditions for these reactions. (Tyrosine showed a slow secondary reaction, resulting in a small absorbance decrease.) All compounds gave slightly higher absorbances (~10%) in the presence of the surfactant compared with the final absorbance in its absence.

Table I—Micellar Catalysis of Amine–Fluorodinitrobenzene Reactions by Cetrimonium Bromide

Amine	Half-Life, min[a]	
	No Surfactant	0.035 *M* Surfactant[b]
Glycine	37.2	2.0
Alanine	85.0	4.7
Phenylalanine	32.0	0.5
Tyrosine	22.0	<0.5
Glycylglycine	63.5	5.4
Glycylglycylglycine	94	7.5

[a] Using 5×10^{-4} *M* amine, 3.5×10^{-3} *M* fluorodinitrobenzene, pH 9.2, 23°. [b] About twice the concentration recommended in the analytical procedure.

The following analytical procedure was developed. To a 25-ml volumetric flask were added 0.12 g of cetrimonium bromide, 1.0 ml of 1.3% (v/v) 1-fluoro-2,4-dinitrobenzene in acetone, 9.0 ml of 2.5% sodium borate in water, and 10.0 ml of an aqueous amine solution (1×10^{-4}–1×10^{-3} *M*). After about five half-lives (Table I) at room temperature, 1.0 ml of the solution was pipetted into 9.0 ml of a 1:100 dilution of concentrated hydrochloric acid in dioxane. The absorbance was read in a 1-cm cell at the absorption maximum against a reagent blank carried through the same procedure.

The absorption maximum was at 340 nm for the tyrosine derivative and at 350 nm for the other samples in Table I. Calibration plots were linear over the sample concentration range.

According to Table I, the more hydrophobic reactants (the aromatic amino acids) gave much larger relative rate enhancements, as expected for micellar catalysis. All rate enhancements are analytically useful, and this method can be applied with the reaction carried out at room temperature. This work provides the scope for further study of this analytical system and of the potential applications of micellar catalysis to other analytical reactions.

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Received May 10, 1979.
Accepted for publication August 21, 1979.
Supported in part by a grant from The Upjohn Co.

Desmethyldiazepam: A Specific Radioimmunoassay

Keyphrases □ Desmethyldiazepam—radioimmunoassay □ Radioimmunoassay—desmethyldiazepam □ Diazepam, desmethyl metabolite—radioimmunoassay □ Benzodiazepines—desmethyldiazepam, radioimmunoassay

To the Editor:

Desmethyldiazepam, a 1,4-benzodiazepine, has particular clinical significance because it is a pharmacologically active metabolite of diazepam and several other benzodiazepines (1). Thus, blood levels of desmethyldiazepam and the parent compounds are important parameters in the pharmacokinetic understanding of anxiolytic drug therapy and in the biopharmaceutical evaluation of different drug formulations.

Although many analytical procedures, primarily employing electron-capture GLC and high-performance liquid chromatography, have been reported for the quantitation of desmethyldiazepam in blood or plasma, none achieves the high sample throughput attainable with radioimmunoassay in which the compound may be measured directly in microsamples. Radioimmunoassay is particularly advantageous in bioequivalency studies, where numerous samples may be generated, and in pharmacokinetic studies in the neonate, where a limited sample volume is available for analysis. We now report the development of a specific radioimmunoassay for desmethyldiazepam which satisfies the requirements for both high throughput and small samples.

The hapten 7-chloro-1,3-dihydro-5-(4-hydrazinocarbonylmethoxyphenyl)-2*H*-1,4-benzodiazepin-2-one was converted to its reactive acyl azide with nitrous acid and was coupled covalently to bovine serum albumin as described previously (2). The immunogen consisted of 15 moles of hapten covalently coupled to 1 mole of albumin. Rabbits were immunized intradermally, and the antiserum with the highest titer (1:7500 dilution) of antibodies to desmethyldiazepam was used for all studies.

The radioligand 9-^{3}H-desmethyldiazepam was prepared by catalytic exchange of 9-iododesmethyldiazepam with tritium gas. The reaction was carried out in tetrahydrofuran containing 0.5% triethylamine with a 10% palladium-on-charcoal catalyst. After removal of the labile tritium by repeated freeze drying from methanol, the reaction product was chromatographed on a silica gel column packed in ethyl acetate. The appropriate fractions were combined to yield 9-^{3}H-desmethyldiazepam with a specific activity of 26.5 Ci/mmole.

The radioimmunoassay was identical to that described recently for diazepam (3). A logit-log calibration curve for desmethyldiazepam was linear from 30 to 2000 pg/tube; thus a working sensitivity limit of 3 ng/ml was achieved using a 10-μl plasma sample. This sensitivity is comparable to that achieved by electron-capture GLC with a 1-ml sample. The intra- and interassay coefficients of variation did not exceed 6 and 11%, respectively.

The specificity of the antiserum was evaluated by cross-reactivity studies with those drugs and/or their metabolites of which desmethyldiazepam is a known metabolite. Diazepam, chlordiazepoxide, desmethylchlordiazepoxide, demoxepam, prazepam, and medazepam all cross-reacted less than 1% relative to desmethyldiazepam (100% cross-reactivity). Clorazepate exhibited an apparent cross-reactivity of >50%, which undoubtedly was due in part to its decomposition to desmethyldiazepam during the assay incubation period. Tricyclic antidepressants, commonly prescribed in conjunction with benzodiazepines, did not cross-react with the antiserum.

Further evidence for the specificity of the radioimmunoassay was obtained by comparison with an established electron-capture GLC method for the determination of desmethyldiazepam (4). The joint determinations of desmethyldiazepam in 30 plasma samples from subjects who had received various doses of clorazepate or diazepam were subjected to linear regression analysis by a method (5) using a 95% confidence ellipse. Over a nearly 300-fold desmethyldiazepam concentration range (24–6400 ng/ml), the correlation coefficient, regression line slope, and y-intercept were 0.998, 1.07, and −5.8, respectively. Thus, the radioimmunoassay measures desmethyldiazepam as precisely and specifically as the electron-capture GLC method.

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Received July 16, 1979.
Accepted for publication August 13, 1979.

0022-3549/79/1100-1471$01.00/0