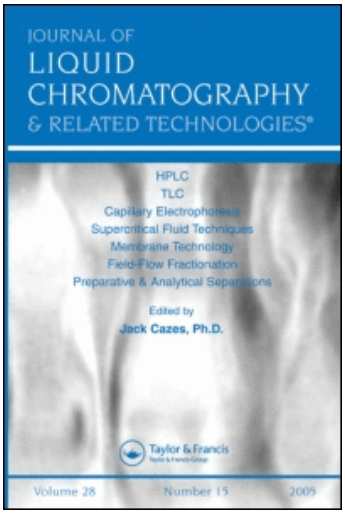

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Development of a Chromatographic Method of Analysis for Glucosyl-Amines Formed from Dapsone

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To cite this Article Parkin, John E. and Boddy, Michael R.(1998) 'Development of a Chromatographic Method of Analysis for Glucosyl-Amines Formed from Dapsone', Journal of Liquid Chromatography & Related Technologies, 21: 14, 2131 — 2142

To link to this Article: DOI: 10.1080/10826079808006613

URL: http://dx.doi.org/10.1080/10826079808006613

J. LIQ. CHROM. & REL. TECHNOL., 21(14), 2131–2142 (1998)

DEVELOPMENT OF A CHROMATOGRAPHIC METHOD OF ANALYSIS FOR GLUCOSYL-AMINES FORMED FROM DAPSONE

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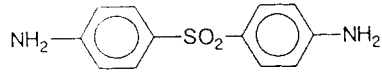
ABSTRACT

A high performance liquid chromatrographic method has been developed for the analysis of dapsone and the five possible mono- and diglucosylamines formed by the reaction of dapsone with glucose. The identity of these glucosylamines has been elucidated by chemical interconversion, by spectroscopic means, and by assessment of their relative abundances at equilibrium. A novel method has been developed to enable the mono- and diglucosylamines to be quantitated using dapsone as an external standard.

INTRODUCTION

Primary aromatic amines are known to react readily under mildly acidic conditions with reducing sugars such as glucose to form glucosylamines (GA's).[1] An investigation of the reaction of procainamide hydrochloride with glucose to form GA's utilising high performance liquid chromatography (HPLC) and nuclear magnetic resonance spectroscopy has been completed in these laboratories.[2] In this study it was demonstrated that the GA's are formed



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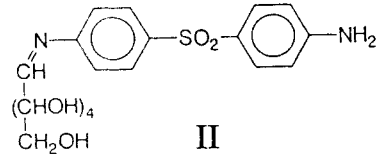

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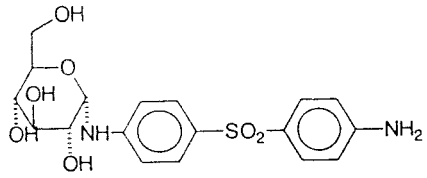

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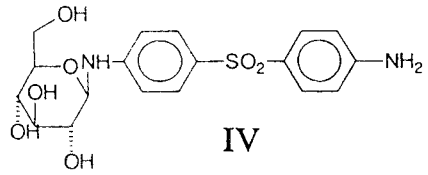

IV

Figure 1. I: dapsone; II: imine formed between glucose and dapsone; III: α-mono-GA; IV: β-mono-GA.

readily under conditions encountered when the drug is admixed with glucose infusion. It appeared possible, therefore, that other drugs with a primary aromatic amino-group may react with glucose or reducing sugars should the drug and carbohydrate be taken concurrently by the oral route and that this reaction might be sufficient to affect the bioavailability. Dapsone (Fig. 1 I), which is used for the treatment of leprosy and as an antimalarial, was chosen as

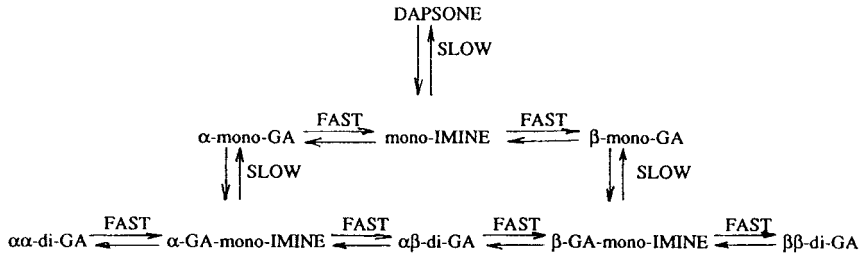

Figure 2. Reaction pathway for the formation of the mono- and di-GA's of dapsone and glucose via their respective imines.

a model compound to test this thesis. The nature of the reaction of primary aromatic amines with glucose has been extensively investigated and for para-substituted aromatic amines it has been consistently demonstrated that an equilibrum is rapidly established with the more stable β-anomer predominating over the α-anomer in the ratio of 9:1.[2-7]

The reaction of dapsone with glucose would be expected to occur via an intermediate imine (Fig. 1, II) to form an acid catalysed equilibrium between the α- (Fig. 1 III) and β-GA (Fig. 1 IV); the intermediate imine not being present in appreciable concentrations and the GA's existing in the cyclic glucopyranosyl forms with the β-anomer predominating in the ratio of 9:1. As dapsone has two identical aromatic amino-groups and is a symmetrical molecule, it should react sequentially with glucose to form, initially, the two anomeric α- and β-mono-GA derivatives which would in turn react further to form three possible di-GA's- αα-, αβ- and ββ-GA (Fig. 2). This paper reports the identification and the development of analytical methods for the quantitation of dapsone and the five possible GA derivatives by HPLC preliminary to an investigation of the reactivity of dapsone with glucose under the influence of acid catalysis.

EXPERIMENTAL

Materials

Glucose-1-hydrate (Ferak Lab., Berlin, Germany) and dapsone (Sigma Chemical Co., St. Louis, MO, USA) were used as supplied. All other chemicals were analytical reagent grade and solvents were HPLC grade.

Chromatographic Equipment and Conditions

The liquid chromatograph consisted of a Model 501 pump (Waters Assoc., Milford, MA, USA), Rheodyne Model 7125 loop injector (Cotati, CA, USA), Model 484 variable-wavelength absorbance detector (Waters Assoc.) and Model 3396A integrating recorder (Hewlett-Packard, Palo Alto, CA, USA), together with a column of octadecyl silica 10 μm particle size, 30 cm x 3.9 mm ID (Phenomenex, Torrance, CA, USA).

The mobile phase was methanol-water (20:80) containing ammonium acetate (0.02M) at a flow-rate of 1.5 mL min^{-1} with a monitoring wavelength of 290 nm and an injection volume of 20 μL.

Spectra of the individual peaks were obtained by use of a Model 991 photodiode-array absorbance detector (Waters Assoc.).

Validation of Analytical Method

The HPLC assay was applied to solutions containing 0.08, 0.16, 0.4, 0.8, 1.2, 1.6, and 2 x 10^{-4} M dapsone in water. The relative standard deviation was determined from six replicates at a concentration of 0.4 x 10^{-4} M. Limits of detection were obtained by sequential dilutions of these solutions.

Preparative Chromatography of Glucosylamines

A solution of dapsone (0.1% w/v) in 0.05 M phosphate buffer pH 3.0 containing acetonitrile (10% v/v) and glucose (10% w/v) was allowed to stand at room temperature for 48 hours. Upon chromatography the GA fractions were collected into vials containing 100 μL of 0.5 M phosphate buffer pH 8.0 and stored under refrigeration for a maximum of 8 hours.

Hydrolysis of Glucosylamines

The vials containing the pure GA fractions were diluted with either 2 M hydrochloric acid or 2 M acetic acid (200 μL). The solutions were mixed and assayed at 1 minute and then at regular intervals for 2 hours. In the case of the hydrolysis with acetic acid the temperature of the solution was raised to 40°C after 1 hour.

Determination of Correction Factors for Quantitation of the Mono- and Di-Glucosylamines

A solution of dapsone (2×10^{-4} M) in 0.05 M phosphate buffer pH 3.0 containing glucose (10% w/v) was stored at room temperature and assayed at 1 hour intervals for 8 hours and then at 48 hours.

RESULTS AND DISCUSSION

All attempts to induce the formation of pure crystalline samples of the glucosylamines formed between dapsone and glucose proved unsuccessful due to the complex nature of the resultant reaction mixture. However, chromatographic conditions were readily found which separated the five possible GA products from dapsone (Fig. 3).

The five peaks (I-V) in Fig. 3 were assigned to the five possible mono- and di-GA's as I: ββ-GA, II: αβ-GA, III: αα-GA, IV: β-GA and V: α-GA on the basis of spectral, chromatographic and kinetic evidence.

Spectral interpretation of the five peaks using a photodiode-array spectrophotometric detector demonstrated that all five peaks had qualitatively similar spectra to that of dapsone with minor spectral shifts. Peaks I-III had λ_{max} at 298 and 262 nm, peaks IV and V at 296 and 260 nm, and dapsone (peak VI) at 292 and 257 nm. The earlier eluting peaks (I-III) would be expected to be due to di-GA's in reverse-phase HPLC due to the higher polarity of these compounds followed by the mono-GA's.

The individual members of each class of GA's, the two mono-GA's and three di-GA's would have identical absorptivity at the monitoring wavelength (290 nm) and therefore peak areas would reflect accurately the relative proportion of isomers present. Capon and Connett (3,4,6,7) have studied the acid-catalysed equilibration of a variety of para-substituted aryl-GA's by polarimetry and have demonstrated that the β-anomer predominates, existing at equilibrium at approximately 90% in the anomeric mixture. Nuclear magnetic resonance studies of the GA's of the analogous para-substituted primary aromatic amine, procainamide, demonstrated that the ratio of α- to β-GA at equilibrium was 12% to 88%.[2] For dapsone, based on peak areas, the ratio was found to be identical - 88% β-GA and 12% α-GA. This strongly supports the interpretation of the identity of the α- and β-GA isomers.

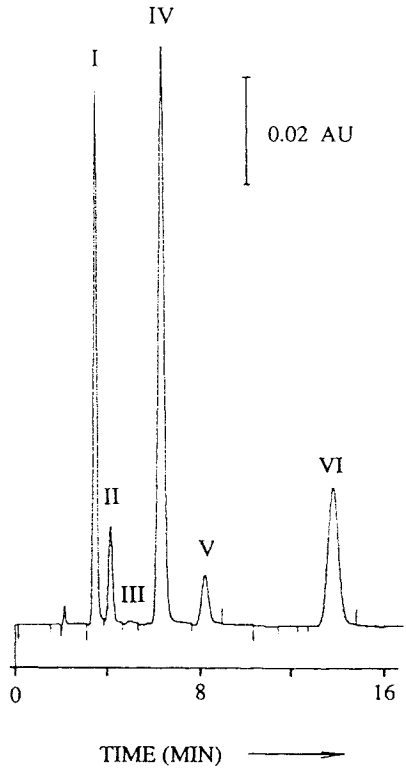

Figure 3. Typical chromatogram obtained following the reaction of dapsone (1 x 10^{-4}M) with glucose (5% w/v) at pH 3.0 after 48 hours at 37°C. I: ββ-di-GA; II: αβ-di-GA; III: αα-di-GA; IV: β mono-GA; V: α-mono-GA; VI: dapsone.

Assuming no stereochemical interference between the two ends of the dapsone molecule the proportion of di-GA's can be calculated from the proportion of mono-GA's. These are 77.4% ββ-GA, 21.1% αβ-GA, and 1.4% αα-GA. The proportion, based on peak areas of the three di-GA's found was 77.0% ββ-GA, 21.5% αβ-GA, and 1.5% αα-GA. This is in reasonable agreement considering that some steric interactions may be involved in some of the isomers.

To confirm these assignments pure fractions of peaks I-V were collected into pH 8 phosphate buffer. Under these conditions GA's were shown to be chemically stable, do not hydrolyse, and be stored and submitted to subsequent hydrolytic investigations by addition of appropriate amounts of acid.[2]

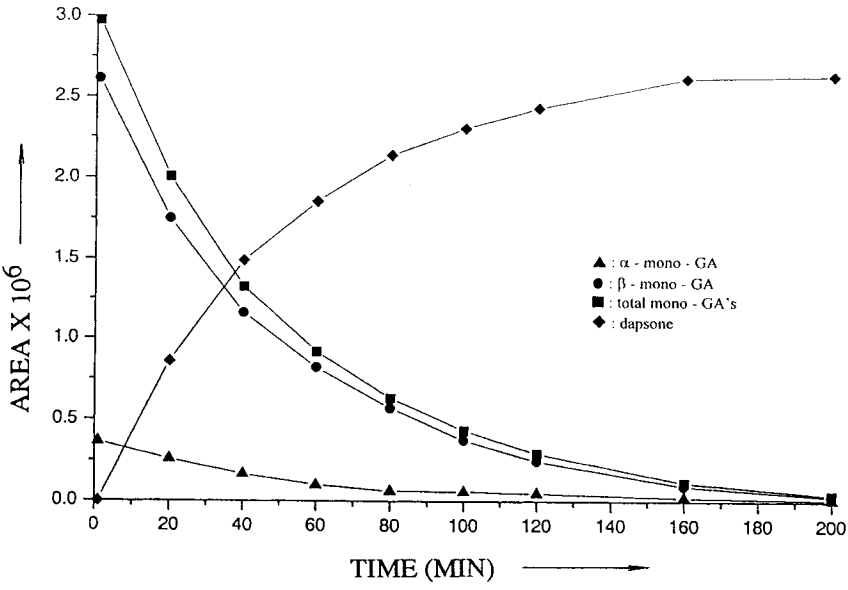

Figure 4. Area response versus time for the hydrolysis of β-mono-GA at pH 1.6.

Addition of hydrochloric acid afforded a solution with a pH of approximately 1.6. When hydrolysis was performed on fractions IV and V it was demonstrated that within one minute the equilibrium was established between the α- and β-GA derivatives (12:88) and that the mixture then underwent hydrolysis more slowly to dapsone (Fig. 4). No evidence of di-GA's (peaks I-III) were apparent during the course of the reaction. This demonstrates that the two peaks IV and V are the mono-GA's with peak IV being the major β-GA. Figure 4 displays the results of the β-GA fraction but similar results were obtained when the α-GA fraction was submitted to hydrolysis under the same conditions.

When the two major di-GA fractions were submitted to the same experiment the equilibrium was established between the ββ-, αβ-, and αα-GA within one minute (77: 21.5: 1.5). The di-GA's underwent rapid hydrolysis to the mono-GA mixture which in turn underwent further hydrolysis to dapsone and glucose (Fig. 5). This figure displays the results of the αβ-GA but qualitatively similar results were obtained for the ββ-GA fraction. This unequivocally demonstrates that the peaks I-III were due to the di-GA's and the relationship between these compounds and the mono-GA's.

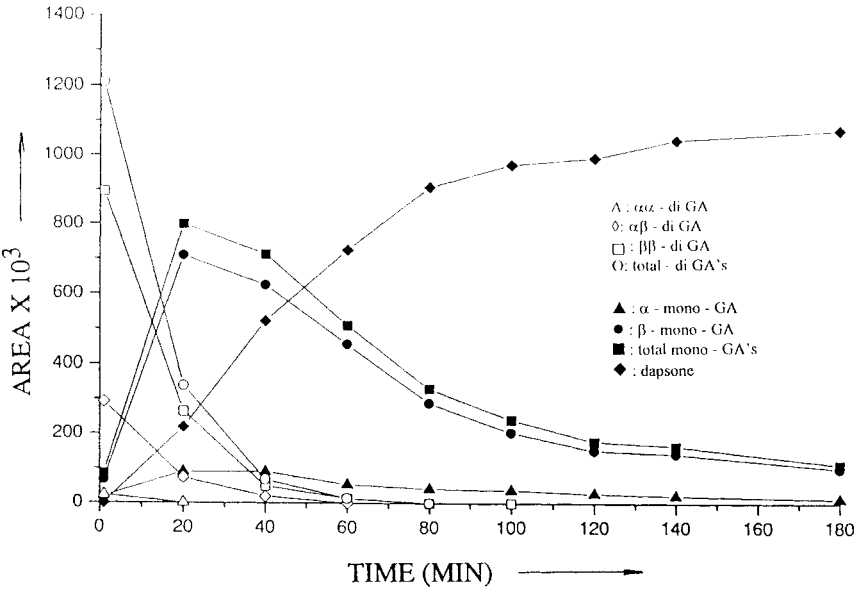

Figure 5. Area response versus time for the hydrolysis of αβ-di-GA at pH 1.6.

To confirm the identity of the individual di-GA's a sample of the αα-GA fraction was submitted to hydrolysis under milder conditions by the addition of acetic acid to afford a pH of 4.2. The sample of αα-GA, being present at low concentration and eluting immediately after the αβ-GA resulted in the sample being contaminated with approximately 5% of the αβ-GA (Fig. 2). This did not influence the interpretation of the result. Under these conditions the anomerisation via the imine proceeded slowly and could be followed chromatographically (Fig. 6A). The ββ-GA was formed in excess with the αβ-GA being formed as a transient intermediate. This confirms that peak II is the intermediate αβ-GA. There was a slow total loss of di-GA's due to hydrolysis to the mono-GA's. Also, for the first hour there was an initial excess formation of α-GA over β-GA unequivocally demonstrating the inter-relationship between the αα-GA and the α-GA (Fig. 6B). This excess of α-GA arises from the fact that initially the concentration of αα- and αβ-GA's exceeds that of the ββ-GA so that hydrolysis via the α-GA-mono-imine is favoured, the reaction being under kinetic rather than thermodynamic control (Fig. 2). This was ultimately followed by the establishment of the expected equilibrium between α- and β-GA and subsequent hydrolysis to dapsone and glucose.

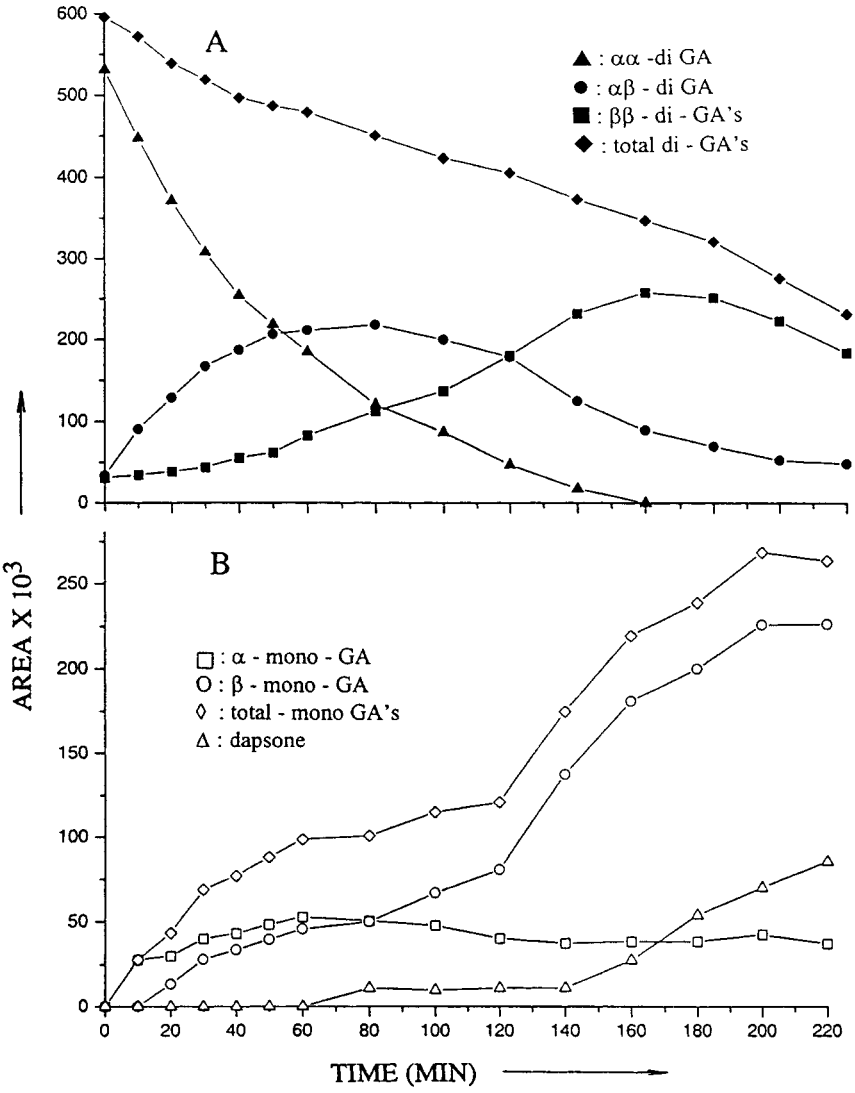

Figure 6. Area response versus time for the hydrolysis of αα-di-GA at pH 4.2.

This evidence unequivocally demonstrates the identities of all of the mono- and di-GA's based on the known identity of the β-GA as the major product at equilibrium of monoglucosylation of primary aromatic amines.

Studies were then undertaken to put the chromatographic separation on a quantitative basis. The assay was validated for dapsone over the range 0.08 - 2 x 10^{-4} M affording a linear response which passes through the origin ($r = 0.9999$, $n = 7$) and a relative standard deviation of ± 0.79% at a concentration of 0.4 x 10^{-4} M. The limit of detection was 10^{-7} M at which the signal to noise-ratio was 3.

There was no chromatographic evidence of any reaction products formed other than the mono- and di-GA's. Capon and Connett[4,7] also found no evidence of significant levels of the intermediate imine on the basis of kinetic evidence when simple aromatic amines were reacted with glucose. Also, no evidence was found of this species when the analogous procainamide was reacted with glucose when the reaction products were studied by nuclear magnetic resonance spectroscopy.[2] To be able to accurately quantitate the GA's it was considered appropriate to determine the absorptivities relative to dapsone of the mono- and di-GA's at the monitoring wavelength of 290 nm so that dapsone could be used as an external standard for these compounds. For the purposes of this work the sum of the areas of the mono- and di-GA's was employed.

During the time-course of the reaction of dapsone with glucose the total molar concentration of dapsone as unreacted dapsone and GA's remains constant. Therefore, at any time during the course of the reaction:

$$A_D - A_{Dt} = A_{mono\text{-}GA}\, f_{mono\text{-}GA} + A_{di\text{-}GA}.f_{di\text{-}GA}$$

where:

A_D = original area of the dapsone peak (zero time).

A_{Dt} = area of dapsone peak at time t.

$A_{mono\text{-}GA}$ = sum of the areas of mono-GA peaks at time t.

$A_{di\text{-}GA}$ = the sum of the areas of the di-GA peaks at time t.

$f_{mono\text{-}GA}$ = factor reflecting the relative absorptivity of the mono-GA to dapsone.

$f_{di\text{-}GA}$ = factor reflecting the relative absorptivity of the di-GA to dapsone.

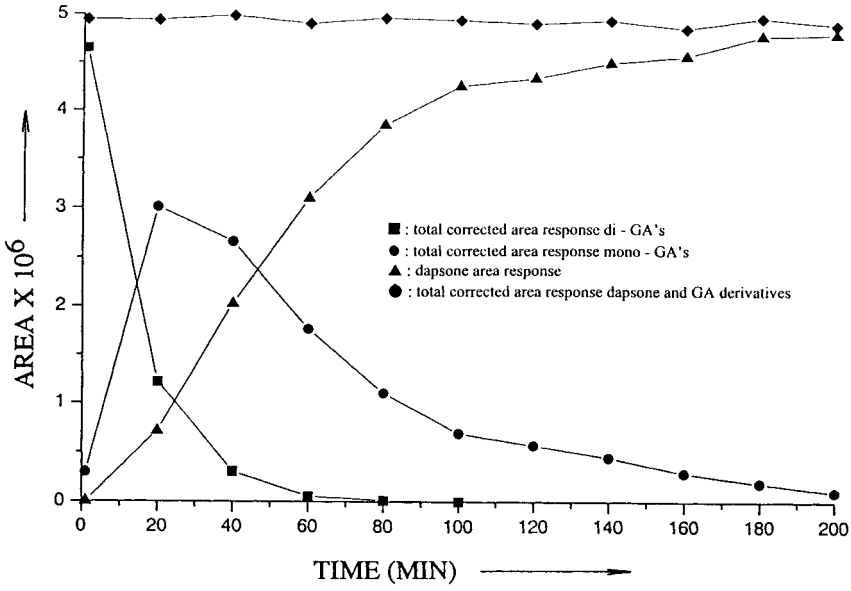

Figure 7. Hydrolysis of ββ-di-GA at pH 1.6 with the peak areas corrected for the absorptivity of the mono- and di-GA's relative to dapsone.

By performing reactions between dapsone (2×10^{-4} M) and glucose (10% w/v) and determining the peak areas of the dapsone and reaction products at times when the concentration of the initially formed mono-GA's were greatest relative to that of the di-GA's, and at equilibrium when the di-GA's were at their maximum concentration, it was possible to construct simultaneous equations which could be solved for $f_{mono\text{-}GA}$ and $f_{di\text{-}GA}$. These values were found to be 0.9077 (± 1.3%, n = 6) for $f_{mono\text{-}GA}$ and 0.7957 (± 1.6%, n = 6) for $f_{di\text{-}GA}$. Using these factors it was possible to translate observed peak areas of the GA's into concentrations relative to dapsone.

That this approach was valid, was demonstrated by re-processing the data for the hydrolysis of the ββ-GA expressing the dapsone formed, the mono-GA's, and the di-GA's in terms of corrected peak areas relative to dapsone (Fig. 7). In this experiment there was a rapid change in concentration of all three components over time but the sum of the areas corrected for the relative absorptivities of the three components remained constant indicating that the correction factors are valid and correct. Dilution of the terminal solutions with pH 8 phosphate buffer demonstrated that for all of the GA's there was a linear

response for peak area versus concentration ($\beta\beta$-GA: r = 0.9990; $\alpha\beta$-GA: r = 0.9989; $\alpha\alpha$-GA: r = 0.9725; β-GA: r = 0.9998; α-GA: r = 0.9992; all n = 5. The low value of r for the $\alpha\alpha$-GA reflects the low initial concentration as it was formed in only small amounts. As the $f_{mono\text{-}GA}$ and $f_{di\text{-}GA}$ values were less than unity the GA's absorb more strongly than dapsone itself at 290 nm and therefore the limits of detection are comparable or lower than that of dapsone.

The assay has been applied successfully to the study of the kinetics of the reaction between dapsone and glucose under the influence of acid catalysis and this will be reported in the subsequent paper.

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Received August 1, 1997
Accepted October 16, 1997
Manuscript 4250