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## A rapid method of monitoring the acute phase response in a rat model

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The acute phase response (APR) is accompanied by major changes in micronutrient status. It is important to be able to quantitate the degree of APR, which can then be related to the accompanying alterations in micronutrient levels. A rapid and convenient means of achieving this, viz. the use of an animal model in which APR can be induced, would facilitate such an investigation.

The aim of the present study was to establish, by means of a rat model, a rapid procedure to identify APR which is less time-consuming and less expensive than the more traditional enzyme-linked immunosorbent assay and radio-immunoassay.

Blood was drawn immediately prior to, and at 24, 48, 72 and 96-hour intervals post-inducement of the acute phase. The serum was deproteinised and treated with a dye, Auramine O, which specifically binds the acute phase protein  $\alpha_1$ -acid glycoprotein (AAG), the latter remaining in solution after deproteinisation. The product formed is a fluorescent addition compound. With fluorescence spectrophotometry, levels of AAG were determined and the extent of APR monitored.

A progressive and reproducible increase in AAG was observed, the highest fluorescence intensity recorded after 48 hours; this indicated a mean value ( $N = 5$ ) of 9.4 mg/ml from zero at baseline. With a spiking approach, a mean AAG recovery of 96% was obtained.

The validated methodology, less expensive and less time-consuming than existing procedures, which rapidly detects AAG in rat serum, provides a simple technique for monitoring the APR process in these animals.

*S Afr Med J* 1997; **87**: 100-102.

Following inflammatory stimuli (e.g. tissue injury or infection), a number of important biochemical changes, known collectively as the acute phase response (APR), take place. This response includes major changes in both macronutrient and micronutrient status, which have made it difficult to interpret blood micronutrient concentrations in acutely ill patients. It is important, therefore, to be able to quantitate the extent and degree of the APR which can then be related to accompanying alterations in micronutrient levels. Among the changes that occur are those in the serum levels of several

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proteins originating in the liver; these are known as the acute phase proteins. The extent of the APR is quantitatively reflected by these changes which may be used to monitor its severity. Thus, we aimed to develop a rat model in which determination of the acute phase protein,  $\alpha_1$ -acid glycoprotein (AAG), would form the basis of such a monitor.

Determination of human acute phase protein levels *in vitro* and *in vivo* is often based on the use of enzyme-linked immunosorbent assay (ELISA) techniques, but mostly involves radial immunodiffusion or immuno-electrophoresis that requires specific antibodies. For similar studies that involve animals, such antibodies are not commercially available and therefore have to be experimentally produced, an expensive and time-consuming exercise. To avoid this, we sought a more convenient method of determining AAG levels in rat plasma.

A previous report<sup>1</sup> on the binding of basic drugs to human AAG describes a rapid and simple fluorimetric technique for the determination of AAG concentrations in serum. Our objective was to apply a similar procedure to plasma drawn from rats at regular intervals following the experimental induction of the APR.

## Materials and methods

Purified rat AAG was obtained from Sigma Chemical Co., St Louis, USA, and 2 mg were dissolved in 0.13 M potassium phosphate buffer (pH 7.4) (400  $\mu$ l). Four aliquots of 6  $\mu$ l, 40  $\mu$ l, 75  $\mu$ l and 120  $\mu$ l were each made up to 200  $\mu$ l with the same buffer and used to establish a calibration plot of fluorescence intensity against concentration, using a Perkin-Elmer model 204A fluorescence spectrophotometer.

Auramine O (Colour index no. 41000) was obtained from Merck, Darmstadt, Germany, and a 0.5 mM solution prepared in ethanol (1.58 mg/ml). Sulphosalicylic acid (BDH Ltd, Poole, England) was used as a protein precipitant (6% weight/volume in water). Heparin sodium solution containing 5 000 IU/ml (Intramed, Port Elizabeth, South Africa) was used to prevent clotting of freshly drawn blood and consequent blockage of the syringe needle. 0.13 M potassium phosphate buffer (pH 7.4) (100 ml) mixed with 2 M sodium hydroxide (1.65 ml), to give solution A, was freshly prepared for each assay. Oil of turpentine was obtained from Heyns Mathew, Cape Town, South Africa.

Three groups of five male Wistar rats (approximately 250 g) from an inbred colony (Medical Research Council) were confined in three plastic cages, in an air-conditioned and humidity-controlled room at approximately 22°C (12-hour light/dark cycle) and with continuous free access to food (standard pellet diet obtained from Specialist Animal Feeds, Cape Town) and water. Each group of rats was successively subjected to the following experimental protocol.

Blood (approximately 1 ml) was withdrawn from the tail vein with a 0.5 ml plastic disposable insulin syringe (29-gauge needle, 13 mm long) containing heparin sodium solution (1 - 2  $\mu$ l). The blood samples (in Eppendorf tubes) were chilled on ice and immediately centrifuged (Microfuge, Beckman Instruments, Cape Town) at 4°C. Plasma was removed and stored at -20°C until required for analysis. Oil of turpentine (1 ml) was injected subcutaneously, as two equal aliquots into two sites in the dorsolumbar region, so as to induce an inflammatory response;<sup>2</sup> subsequent blood samples were similarly drawn at 24, 48, 72 and 96-hour intervals post-injection and treated as already described.

Aliquots (200  $\mu$ l) of the different standard solutions or rat plasma were treated with 6% sulphosalicylic acid solution (200  $\mu$ l), stirred thoroughly for 10 seconds on a vortex mixer, placed on ice for 5 minutes and centrifuged for 5 minutes. Supernatant (300  $\mu$ l) was transferred to small glass tubes containing solution A (2 ml) and stirred as described above. The mixture was carefully transferred to a cuvette and the baseline fluorescence was measured (excitation and emission wavelengths 470 and 550 nm, respectively). Auramine O in ethanol (40  $\mu$ l) was added to the contents of each cuvette and carefully mixed. Fluorescence intensity was again measured using the same wavelength settings. A calibration plot based on the difference in fluorescence intensity against AAG concentration was established (Fig. 1) with the standard solutions, and used to determine levels of AAG in plasma samples.

Two 200  $\mu$ l aliquots of rat plasma were spiked with phosphate buffer solution of AAG — 40  $\mu$ l; 0.2 mg and 75  $\mu$ l; 0.375 mg, respectively. These spiked plasma samples, along with an aliquot (200  $\mu$ l) of the corresponding unspiked plasma, were taken through the procedure as described above. Difference in fluorescence intensity was recorded (Table I), together with corresponding observed weights of AAG in the samples. The latter were then corrected for a sample volume of 200  $\mu$ l and the weights of AAG in the two spiked samples recovered were calculated from their difference from the unspiked sample. The values thus obtained for the AAG recovered were then compared, in each case, with the weights of AAG originally added, after which the recovery was calculated.

## Discussion

Human AAG has been shown to bind basic drugs in serum and has been the subject of studies on interpretation of their pharmacokinetic data.<sup>3,4</sup> Additionally, several reports have described the importance of measuring AAG levels and methods for doing so.<sup>5-8</sup> The use of a fluorescent probe to obtain information on the interaction between proteins and small molecules<sup>9,10</sup> is based on the observation that an aqueous solution of the probe, when added to a solution of a major binding protein, results in the formation of an

**Table I. Recovery of AAG from rat serum**

AAG spike added (mg)	Sample volume ( $\mu$ l)	Fluorescent intensity (WT. AAG mg)	Corrected weight AAG (mg/200 $\mu$ l)	Calculated spike (mg)	Recovery %
—	200	8 (0.140)	0.140		
0.2	240	13 (0.405)	0.338	0.198	99
0.375	275	18 (0.665)	0.484	0.344	92

addition compound which fluoresces strongly, whereas the two components individually do not fluoresce or do so only very weakly. This has been extended to the determination of AAG in human serum using Auramine O as the probe.

In addition to AAG, Auramine O reacts with albumin and, to a lesser extent, gamma-globulin, forming the corresponding fluorescent addition compounds. Consequently, before AAG determination, removal of these proteins is necessary; this was achieved by selective sulphosalicylic acid precipitation<sup>1</sup> based on a procedure for isolating AAG from human serum.

The methodology described here has been developed as a rapid and more convenient alternative to more traditional procedures for monitoring the APR in the rats.

## Results

This procedure for AAG determination involves a differential measurement of fluorescence intensity obtained from readings taken before and after the addition of Auramine O to standard solutions of rat AAG. The standard calibration plot of this data (Fig. 1) shows a linear relationship ( $r^2 = 0.982$ ) between rat AAG concentration and differential fluorescence intensity, similar to that previously reported<sup>1</sup> for human AAG, including the same tendency to underestimate AAG concentration, as shown by the negative intercept on the ordinate.

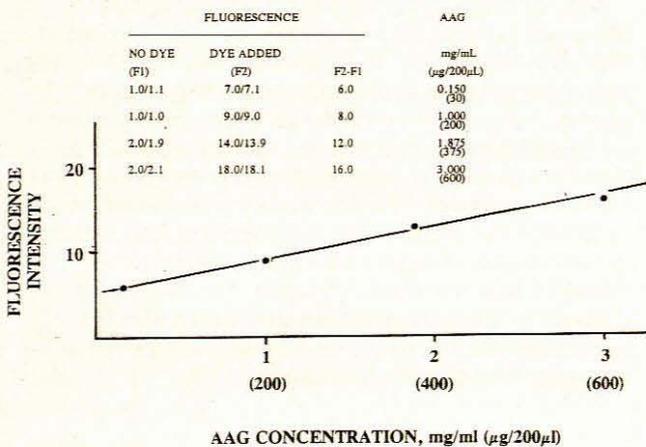


Fig. 1. Standard curve for the measurement of rat AAG using the Auramine O method.

This methodology was then applied to the monitoring of AAG levels in plasma samples drawn from rats immediately prior to their injection with turpentine and again at 24, 48, 72 and 96-hour intervals thereafter. A progressive and reproducible increase in AAG was observed with a maximum level reached between 48 and 72 hours post-injection (Fig. 2). The level of AAG observed in this study (highest mean value 9.4 mg/ml from zero at baseline) is considerably higher than that reported by Schreiber *et al.*<sup>2</sup> in rats (3.2 mg/ml from 0.17 mg/ml at baseline). The latter may be related to the difference in the intensity of the APR induced. The experiment was successively repeated sequentially on two further groups of rats with similar results (Fig. 2), though the one group, in response to the same stimulus, surprisingly produced less AAG than either of the other two.

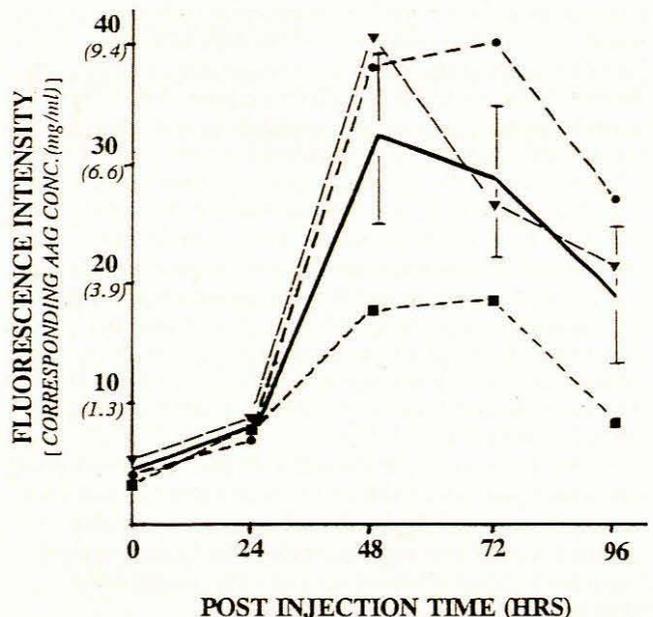


Fig. 2. Fluorescence intensity and AAG levels in rat plasma v. post turpentine injection time. (Each data point is the mean of five values. Overall means from the three experiments are plotted as a solid line with SEM error bars.)

The method was also tested on samples of a rat plasma spiked with rat AAG at two different levels. When compared with corresponding unspiked samples (Table I), the data indicate a mean AAG recovery rate of 96%.

## Conclusion

We have validated a methodology that is convenient, less expensive and less time-consuming than existing procedures, which rapidly detects AAG in rat plasma, thereby providing a simple technique for monitoring the APR process in these animals.

Financial assistance from the MRC is acknowledged.

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