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# High-performance liquid chromatography determination of α1-acid glycoprotein in small volumes of plasma from neonates

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**Abstract**

In order to investigate how the α1-acid glycoprotein (AGP) concentrations of neonates change in response to surgical stress, a simple high-performance liquid chromatography (HPLC)-assay for the measurement of α1-acid glycoprotein levels was developed. A fraction containing α1-acid glycoprotein was isolated from the bulk of plasma protein by addition of 0.6 M perchloric acid and was then analysed directly on a short PLRP-S 4000 Å reversed phase column column. The method was validated by analysis of pooled plasma from healthy adults both in comparison with a calibration curve and by standard additions. The procedure was able to isolate α1-acid glycoprotein rapidly (<30 min) and required only 50 μl of plasma. The mean extraction recovery was 79.1% (CV 6.4%). The within-run precision for the analysis of three replicates of quality control sample ranged from ±1.2 to ±3.8% and the between-run precision was ±6.1%. The method was linear ($r^2 = 0.988$) over a concentration range from 6 to 100.0 mg/100 ml. The AGP levels in neonatal samples ranged from 25 to 93 mg/100 ml.


*Keywords:* Anaesthetics; Alpha1-acid glycoprotein; HPLC; Neonates; Plasma

## 1. Introduction

Alpha-1-acid glycoprotein (AGP, AAG or orosomucoid) is an acute phase protein with a molecular weight of 40,000 Da and unusually high carbohydrate content (40–45%) [1]. With an isoelectric point (pI) of between 2.7 and 3.5, AGP is generally regarded as one of the most acidic proteins and is often associated with the binding of basic drugs. The concentration of AGP in ranges from 40 to 100 mg/100 ml in healthy adults under normal circumstances, but can vary considerably and often changes in response to various stressful stimuli such as surgery [2]. Thus, AGP levels should rise in the post-operative period as has been shown in adults, but not confirmed in neonates.

In neonatal plasma, AGP concentrations are markedly reduced when compared the concentrations observed in adults [3]. For local anaesthetics that are given as post-operative pain relief, the effect (therapeutic or toxic) is principally dependent on their free concentrations. Therefore, knowledge of the factors influencing drug binding, such as AGP levels is critical to the establishment of a safe dosage guideline for these drugs in neonates.

Of the many ways described to isolate AGP, most methods are based on either; the molecules net charge, which, at pH values above 4, is always highly negative because of the very acidic pI; or the fact that it is extremely water soluble near it's pI in comparison with some of the more lipophilic proteins in plasma like human serum albumin (HSA). Therefore, AGP can be relatively easily separated from the other plasma proteins by electrophoretic, ion-exchange chromatography or solubility procedures. The negative charge of AGP has been previously exploited for it separation by using ion-exchange and hydroxyapatite columns [4]. In another study a recovery of 65% of AGP was achieved by affinity chromatography using blue sepharose CL-6B (Pharmacia), which separated AGP from the other plasma proteins [5]. Crude samples of AGP have also been prepared by precipitation of interfering plasma proteins with 6% sulphosalicylic acid [6] or perchloric acid [7]. Other preparations were obtained by extraction of AGP with liquid phenol and chloroform–methanol [8]. When compared with the extraction procedure described in the current paper,

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the methods above use time consuming and laborious extraction protocols.

AGP has been commonly measured by single radial immunodiffusion (SRID), which can take 24–48 h to perform. Methods based on binding the dyes quinaldine red [9] and auramine O [10] have been reported for the determination of AGP. Although these methods are conveniently able to determine AGP without a previous isolation step, they suffer from disadvantages such as long analysis times, lack of sensitivity at low concentrations or the need for large sample volumes. Recently, capillary electrophoresis (CE) has been used to analyse plasma proteins such as AGP. For example, a CE method was developed for the quantification of AGP in cerebrospinal fluid [11]. However, most of these methods are used simply to identify AGP in various plasma fractions [12,13] or to investigate the heterogeneity of glycosylation of the glycoprotein [14] rather than quantify it.

The major alternative to the methods described above is high-performance liquid chromatography (HPLC). HPLC has become widely used for the quantitative analysis of proteins in recent years. Although AGP has been measured in urine by direct injection onto a reversed-phase HPLC column [15,16], the analysis of AGP in plasma by HPLC, as described by [17] and [18], is relatively complex. The latter method applied a solvent extraction followed by microanalytical ion-exchange chromatography for measuring AGP in human serum. In the present study a single step isolation procedure has been developed and coupled with a rapid HPLC method in order to quantify the AGP levels in small volumes of plasma from infants undergoing major surgery. As far as we are aware there is no simple, rapid method available for the quantitative determination of AGP in small volumes of plasma by HPLC.

## 2. Experimental

### 2.1. Chemicals

Human α1-acid glycoprotein, trifluoroacetic acid (TFA; puriss ≥99%), perchloric acid (70%; SLR grade) and human serum albumin were all obtained from Sigma–Aldrich Co. Ltd., (Poole, UK). Water (HiPerSolv$^{TM}$), acetonitrile (HiPerSolv$^{TM}$) and methanol (HiPerSolv$^{TM}$) were purchased from VWR (Lutterworth, Leics., UK). A quality control (QC) plasma sample which had been pooled from healthy donors was obtained from the Blood Donor Centre, Glasgow and stored at −20 °C.

### 2.2. HPLC analysis

The liquid chromatographic system consisted of a SpectraSYSTEM P2000 gradient pump, a SpectraSYSTEM AS3000 autosampler (equipped with a Type 7010-150 Rheodyne injection valve (20 μl loop)) and a SpectraSYSTEM UV1000 detector (Thermo Separation Products, Inc.). The column used was a PLRP-S 4000 Å column (50 mm × 4.6 mm I.D., 5 μm particle size, Polymer Laboratories Ltd, Church Stretton, UK). A gradient consisting of 1 ml TFA/l of water (solvent A) and 1 ml TFA/l of acetonitrile (Solvent B) was used as follows: 0 min 18% B, 15 min 60% B, 17 min 60% B and the column was re-equilibrated for 7 min before the next injection. The flow rate was 1 ml/min. The detection wavelength was set to 220 nm. Data were acquired and processed using ChromQuest software.

### 2.3. Sample preparation and assay

A stock solution of AGP at a concentration level of 2 mg/100 ml was prepared in water and aliquots of 100 μl were stored frozen at −20 °C. Different calibration solutions were prepared by sequentially by diluting the stock solution to give solutions in 0.5 M perchloric acid. The standards (6.25, 12.5, 25.0, 50.0, and 100.0 mg/100 ml) were injected directly into the chromatographic system. For the determination of plasma concentrations of AGP, 50 μl of plasma (control or patient sample) and 100 μl of 0.5 M perchloric acid were vortex mixed in a 0.5 ml Eppendorf tube for 20 s. The acidified plasma was then centrifuged at 3000 × *g* for 20 min at room temperature (Microcentrifuge FORCE 7, Fischer Scientific, UK). One hundred microliters of the supernatant was then transferred to a 200 μl insert in a HPLC autosampler vial. Extracted standards and samples were stored at 4 °C if they could not be immediately chromatographed.

AGP concentration was calculated from the linear correlation of AGP (μg) versus peak area values obtained from the perchloric acid extract. Results are expressed as mg/100 ml of plasma.

### 2.4. Method validation and analysis of patient samples

In order to obtain information on the accuracy of the procedure, the recovery of AGP by perchloric acid extraction was determined. Various amounts of AGP (0, 12.5, 25, and 37.5 μg) were added to a constant volume of plasma (50 μl) and then mixed with perchloric acid as before in order to prepare a standard addition curve. The linearity of the method was established using the standards for AGP dissolved in perchloric acid as described above. Within-run reproducibility of retention time and peak area was examined by repeated injection ($n = 3$) of an AGP standard (0.5 mg/100 ml). Three replicates prepared from the 50 μl amounts of the pooled plasma QC sample were run on three different occasions to calculate within and between run precision. Replicates of the same QC samples were run alongside the patient samples to monitor the performance of each assay.

In order to investigate the clinical relevance of the method, AGP concentrations were quantified in samples of plasma from neonates. With ethics approval and parental consent, seven neonates, whose post-conceptual age is up to 48 weeks, undergoing epidural analgesia were recruited to the study. A maximum of only 1 ml/kg of venous blood was allowed by the hospital ethics committee for the purposes of research and therefore 2–7 blood samples of 1 ml (producing up to 0.5 ml plasma) were taken from each baby. The plasma samples were stored at −20 °C until required.

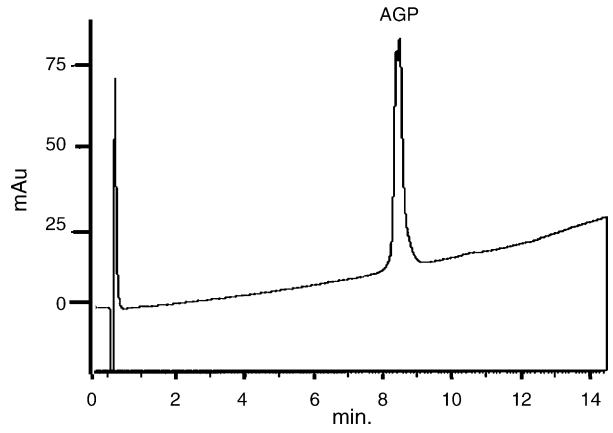


Fig. 1. Unextracted aqueous standard containing 25 mg/100 ml of AGP.

## 3. Results

Fig. 1 shows a typical chromatogram of a solution of the AGP standard in 0.5 M perchloric acid and Fig. 2 shows AGP in a pooled plasma control sample similar chromatograms were obtained from neonatal plasma samples. The elution times for the AGP were between 8.7 and 8.9 min, with any day-to-day variation occurring within these times. Although the peaks obtained are quite broad, the likelihood of confusing AGP with other plasma proteins is minimal, especially since the major plasma protein, HSA, is almost completely removed by the perchloric acid precipitation step. However, an HSA standard in water was run under the current conditions to verify a differing elution time and was found to elute from the system between 7.6 and 8 min. The calibration curves for peak area plotted against concentration for AGP were linear ($r^2 = 0.997$, $y = 31980x - 253955$ $n = 4$, Fig. 3) over a concentration range from 6.0 to 100 mg/100 ml, encompassing the concentrations of AGP in the neonatal plasma samples. Pooled human plasma samples spiked with AGP in a range of 0–75 mg/100 ml, showed a mean recovery of 79.1% (R.S.D.) when the individual points were compared with the average values obtained from the calibration standards. Better calibration curves were obtained by the standard addition

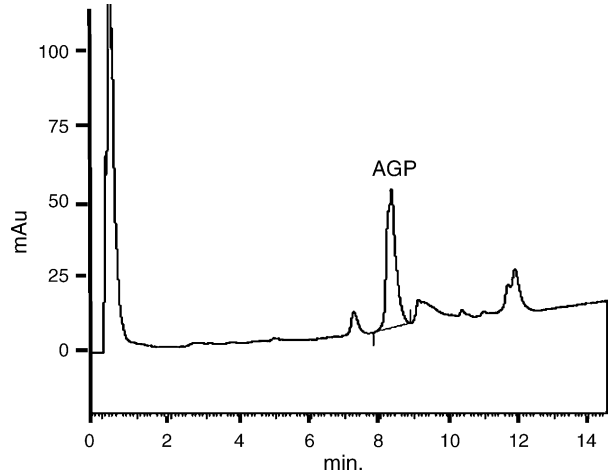


Fig. 2. AGP extracted from 50 μl of control plasma.

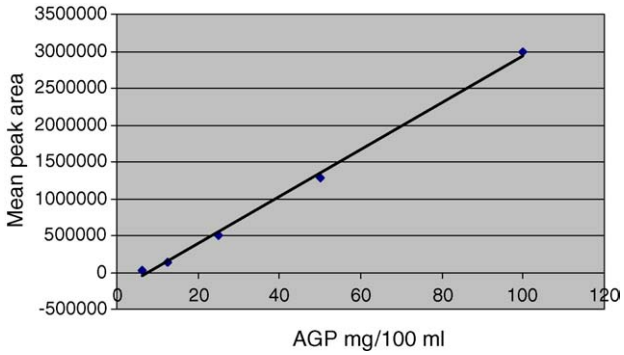


Fig. 3. Calibration curve used for AGP determination.

Table 1
Within and between run precision for the analysis of AGP in 50 μl volumes of control plasma

| | *n* | AGP levels mean (mg/100 ml) | R.S.D. (%) |
|---|---|---|---|
| Within-run | | | |
| Day 1 | 4 | 51.5 | 1.2 |
| Day 2 | 4 | 45.5 | 2.9 |
| Day 3 | 4 | 39.5 | 3.8 |
| Between-run | 3 | 45.5 | 6.1 |

method and this would have been the method of choice for quantification should sufficient plasma have been available. The mean value for AGP in the pooled plasma 45.5 mg/100 ml is within the normal physiological range. As shown in Table 1, the within-run precision of the extraction/HPLC procedure ranged from ±1.2 to ±3.8% and the between-run precision was ±6.1%. The results obtained for a neonatal patient undergoing a single injection of local anaesthetic and for patients receiving a continuous infusion over a longer period are presented in Table 2. The AGP levels of the patient samples were initially mostly either below or at the bottom end of the normal adult range. The AGP plasma concentrations of patients receiving continuous infusions showed significant increases with time. The results were similar to those reported previously [18], which showed an increase in mean AGP concentration from 38 mg/100 ml (preoperative; range 7–78 mg/100 ml) to 76 mg/100 ml by day 4. Similar results were found by Larsson et al. [19], while investigating AGP levels in neonates after continuous epidural infusion. In this study the mean AGP level was 29 mg/100 ml at 1 h and,

Table 2
AGP levels of neonates given continuous infusion anaesthetic for up to 64 h

| Patient | Time (h) (AGP concentration (mg/100 ml)) | | | | | |
|---|---|---|---|---|---|---|
| 1[a] | 0.5 (33) | 1 (33) | 1.7 (32) | 2.3 (31) | 3 (33) | 3.5 (34) |
| 2 | 2 (46) | 6 (47) | 26 (71) | 42 (76) | | |
| 3 | 6 (35) | 13 (42) | 37 (83) | | | |
| 4 | 3 (27) | 20 (29) | 44 (34) | | | |
| 5 | 1 (24) | 6 (28) | 21 (42) | 25 (62) | | |
| 6 | 0.5 (27) | 19 (45) | 41 (74) | 64 (93) | | |
| 7 | 6 (26) | 20 (34) | 49 (34) | | | |

[a] Single bolus injection.

although it had increased to 50 mg/100 ml at 24 h of infusion, it was still at the lower end of the normal adult range.

## 4. Discussion

Previous methods for AGP determination require multiple steps or time-consuming procedures. In order to detect the very low AGP levels of some of our neonate samples with a resolution from any interfering peaks it was necessary to remove serum albumin, which is generally present at a concentration of ca. 4000 mg/100 ml. Since human serum albumin is also an anionic protein and has similar properties to AGP (MW 66000; pI 4.0–5.8), a good resolution could not be achieved by simply changing the gradient conditions of the HPLC method because of the high abundance of HSA in plasma. The initial approach to the problem was to perform a one-step isolation procedure based on methanol–chloroform extraction [8]. The AGP remained dissolved in the methanol–water phase, but the extraction failed to eliminate the HSA completely. Therefore, albumin-free extracts were obtained by precipitating major plasma proteins with perchloric acid. AGP is relatively abundant in plasma and is particularly stable in acidic solution probably because its extensive glycosylation permits it to remain water soluble when unfolded. The present method for the isolation of AGP has several advantages over previously published techniques. Firstly, it can be carried out rapidly and the use of a polymer column means that the perchloric extract can be injected onto the HPLC column without further sample preparation. Secondly, the volume of plasma needed for the extraction and the subsequent quantification of AGP was only 50 μl. The mean recovery of 79.1% for GP estimated from standard addition was lower than might have been hoped for, but recovery improved as more AGP was added in the standard addition was added which reflects the fact that precision of determination is better when: $y = \overline{y}$.

Thus, the lower points on standard addition curve underestimate the AGP content of the plasma. Since sample volumes were limited, since they were also required for determination of the infused anaesthetic, quantification of AGP in the neonatal plasma samples was based on direct calibration rather than standard addition. The isolated protein in the perchloric extract was identified as AGP by comparing its retention time with that of an AGP reference standard.

The separation of proteins on reversed phase columns suffers commonly from practical problems: broad bands which limit overall sample resolution, tailing or distorted bands which complicate quantitative analysis, incomplete recovery, carry-over and splitting of a single protein into two or more distinct bands [20]. Quantitative analysis of AGP was done on a short PLRP-S 4000 Å column from Polymer Laboratories, which is a high efficiency wide-pore polymer based support, particularly manufactured for protein analysis, containing rigid, macroporous spherical particles of polystyrene/divinylbenzene which are stable across the complete pH range which can be used to analyse the very acidic perchloric acid extracts.We observed in earlier work with a 15 cm PLRP-S 300 Å column that there were problems of column contamination since very through washing had to be carried out in order to eliminate traces of plasma proteins adhering to the column (unpublished data). In the current work only a short additional wash period was required with 60% of solvent B after each run in order to eliminate carryover.

The method proved to be sufficiently sensitive to quantify the lowest AGP level in neonate samples (equivalent to an AGP level of 19 mg/100 ml in plasma). We also attempted to measure AGP levels previously [21] by using the above mentioned fluorometric method [10] but the AGP concentrations were below the limit of detection of the method, although not always, as the current study reveals, below the normal range.

In view of the range, precision, sample volume and work time required, the method described in this paper is suitable for performing rapid measurements of AGP in small volumes of plasma. The improvement in sample preparation and analytical conditions enabled us to obtain reproducible and precise results even in small samples of plasma from neonates.

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