Absorption of Some Aminoglycoside Drugs by Barrier Gels in Sampling Tubes, Gregorio Alcantarilla* and Mª Dolores Lozano (Service of Clin. Anal., Hosp. J.R. Jimenez,* 21005 Huelva, Spain; *author for correspondence: fax 34-59-202080)

Blood specimens for drug assays are almost invariably collected in commercially available evacuated tubes. In the past, Vacutainer Tubes (Becton Dickinson, Rutherford, NJ) reportedly [1, 2] decreased the concentration of the basic drugs in samples because of the plasticizer tris(2-butoxyethyl) phosphate in the rubber stopper used with these tubes. The basic drugs were displaced from their protein binding sites, with subsequent uptake by erythrocytes, resulting in spuriously low values for concentrations in plasma [3]. Tubes with stoppers that are stated to be free of this compound have recently become available for specimen collection. Or, one can use tubes for blood collection that contain an inert barrier material (a silicon polymer gel) and a clot activator. The relative density of the gel is intermediate to that of the serum and the coagulum after clotting and centrifugation. After centrifugation, the samples can be transported or stored in the gel tubes until assayed, without first decanting the serum.

Some authors have observed decreases in antiepileptic [4–6] and antiarrhythmic [7] drugs kept over the gel barrier at various temperatures. Here we report our evaluation of the concentration stability of some aminoglycoside drugs—amikacin, gentamicin, and tobramycin—in blood stored on gel separation tubes—specifically, nonanato-coagulated plain red-top 5-mL Venoject® tubes (Terumo Europe S.A.) and 4-mL Vacutainer Serum Separator Tubes (SST®; Becton Dickinson)—after centrifugation.

Blood was sampled into Venoject plain red-top and SST tubes from 10 patients who were taking aminoglycoside drugs. The samples were left upright, undisturbed, for 30 min at room temperature to allow the samples to coagulate and then were centrifuged at 1500g for 10 min. Serum from the Venoject plain red-top tubes was pipetted into polypropylene tubes immediately after centrifugation (reference tubes). The collection tubes were stored at 4°C with 1 mL and 0.2 mL of serum left in contact with gel barrier, and an aliquot of each sample was pipetted into polypropylene tubes after 1 and 2 days. We measured the serum drug concentrations in all of the samples in the same run, using Emit immunosassays (Syva, Palo Alto, CA) and a Cobas-Mira automated analyzer. The average CV of this method for assay of all aminoglycosides was <4.2%.

We observed significant time- and volume-dependent decreases in the measured concentrations of tobramycin and amikacin when the serum was stored at 0–4°C in Vacutainer SST tubes (Table 1). In contrast, under identical conditions, gentamicin did not change significantly. These pronounced differences are presumably related to the binding of the drug to the gel.

The decrease in drug concentration could be reduced by minimizing the interval between centrifugation and decantation. The tube-induced decrease in concentration of these drugs in serum illustrates a potential source of clinically important error in drug measurement that would not be detected by the usual quality-control procedures.

References


Table 1. Effect of storage in SST tubes on concentrations of aminoglycoside drugs in serum.

<table>
<thead>
<tr>
<th>Reference (Venoject) mean conc, μmol/L</th>
<th>Days stored in SST tubes*</th>
<th>1 d</th>
<th>2 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin 13.92</td>
<td>93.2 (0.47)</td>
<td>81.5 (1.29)</td>
<td></td>
</tr>
<tr>
<td>Tobramycin 2.31</td>
<td>80.4 (0.23)</td>
<td>64.1 (0.41)</td>
<td></td>
</tr>
</tbody>
</table>

*Samples collected in Venoject red-top tubes showed no decrease, and the CV was <3.2% in all cases, in drug concentrations during storage at 0–4°C.
**Results shown are mean (SD) for 10 patients, with stored samples of 0.2 mL of serum; no significant differences were seen for stored volume of 1 mL.
***Significantly different from reference value: P <0.05 (Student's t-test).

Stability of Plasma Nonesterified Arachidonate in Healthy Individuals in Fasting and Nonfasting States, Youssef Hallaq, Zbigniew M. Szczepiokowski, Jan Tertuya, Joanne E. Cluette-Brown, and Michael Lapoosta* (Dept. of Pathol., Div. of Clin. Pathol., Massachusetts General Hosp., Fruit St., Boston, MA 02114; *address correspondence to this author at: Rm. 235 Gray Bldg., Massachusetts General Hosp., Boston, MA 02114; fax 617-726-3256, e-mail LAPOSATAMI@A1.mgh.harvard.edu)

Eicosanoids synthesized from arachidonate by platelets and endothelial cells may be provided by albumin-bound nonesterified (free) arachidonate in the plasma, by phospholipid-associated arachidonate liberated from the membranes of platelets and endothelial cells upon cell activation, or by both. If there is no mechanism to limit its plasma concentration and its availability for eicosanoid production, the nonesterified arachidonate in the plasma is capable of stimulating cells to synthesize eicosanoids directly upon incorporation into the cells. The presentation of nonesterified arachidonate to cells can bypass the need for mobilization of cellular arachidonate from phospholipids for eicosanoid production by rapidly entering the cells and being converted to eicosanoids without agonist stimulation.

Because the concentration of total nonesterified fatty acids in plasma is much lower than that of esterified fatty acids, a precise and accurate quantification of total plasma nonesterified fatty acids, particularly nonesterified arachidonate, has long been a matter of uncertainty. The range of reported nonesterified plasma arachidonate concentrations is 0.9–35.5 μmol/L [1–8]. Because of the tedious work required for accurate quantification of individual nonesterified fatty acids, many simplified methods have been developed for measurement of these compounds.
However, as we [9] and others [7] have shown, the advantage of technical ease often results in inaccurate determinations. Contamination of the small nonesterified fatty acid pool with even 1–2% of the much larger pool of esterified plasma fatty acids makes accurate measurement of nonesterified fatty acids in plasma impossible. To permit accurate determination of the nonesterified arachidonate concentration in plasma, we developed an accurate and precise method [9], using conventional techniques, multiple internal standards, and meticulous conditions to avoid fatty acid oxidation. With this methodology in hand, we addressed the question of variability in plasma nonesterified arachidonate between fasting and nonfasting states in 11 healthy subjects. We now report that the concentration of free arachidonate in normal nonfasting plasma is 1.2 ± 0.3 μmol/L (mean ± SD), with a range of normal values from 0.7 to 1.5 μmol/L [9]. This concentration is significantly lower than most of the values previously reported [1–8].

In the present investigation, our goal was to determine the fluctuation in the concentration of plasma nonesterified arachidonate in healthy individuals in nonfasting and fasting states. We found that, as nonesterified fatty acid concentrations in healthy individuals increased in the fasting state to exceed the values in the nonfasting state, the relative increase in arachidonate was the lowest among all the nonesterified fatty acids.

Blood samples were collected into EDTA Vacutainer Tubes (Becton Dickinson, Rutherford, NJ) from 11 individuals in the fasting state (for 8 to 12 h) and subsequently in the nonfasting state. None was taking medications at the time of the study. Total plasma lipids were extracted by using a modified Folch method [10], after which total nonesterified fatty acid was isolated by using thin-layer chromatography (TLC). Nonesterified fatty acids isolated by TLC were methylated with methanol: acetyl chloride (50:1, by vol) and quantified by gas chromatography [7].

The data in Table 1 are the results of nonesterified fatty acid analyses for the 11 individuals in the fasting and nonfasting states. As shown, the concentration of nonesterified arachidonate in the plasma of fasting individuals was 2.2 ± 0.6 μmol/L. The absolute amount of increase (in μmol/L) from the nonfasting to the fasting states was lowest for nonesterified arachidonate among all the fatty acids, in large part because the baseline concentration of nonesterified arachidonate in the plasma was the lowest of all the fatty acids detected (Table 1). Notably, however, with fasting, arachidonate showed the lowest percent increase of all the nonesterified fatty acids. Stearate also showed a relatively small increase. The large standard deviations in the Table most likely reflect the differences between individuals and, possibly, small differences in the length of fasting (8 to 12 h), rather than analytical variability.

The results of our study demonstrate that the physiological concentration of nonesterified plasma arachidonate is quite low, and that the concentration of nonesterified arachidonate in plasma is less influenced by fasting than is any of the other nonesterified fatty acids. The concentration of nonesterified plasma fatty acids approximately reflects dietary fat composition [11–13]. The liberation of fatty acids during lipolysis in the fasting state, which is apparently lower for arachidonate and stearate, is therefore at least partially independent of the type of dietary fat consumed. This observation of low nonesterified arachidonate may also be related to the preferential uptake of arachidonate by cells, which could reduce the plasma concentration of nonesterified arachidonate. There is substantial evidence for preferential uptake of arachidonate by cells [14], but not for stearate. The basis for the relative stability in the plasma concentrations of both nonesterified arachidonate and nonesterified stearate remains to be determined.

### Table 1. Mean ± SD concentrations of plasma nonesterified fatty acids in healthy individuals.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Nonfasting</th>
<th>Fasting</th>
<th>Absolute increase, μmol/L</th>
<th>% Increase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:4n-6</td>
<td>1.2 ± 0.3</td>
<td>2.2 ± 0.6</td>
<td>1.0 ± 0.6</td>
<td>92.2 ± 76.5</td>
</tr>
<tr>
<td>13:0</td>
<td>2.3 ± 1.5</td>
<td>6.4 ± 3.7</td>
<td>4.1 ± 3.5</td>
<td>250.3 ± 236.5*</td>
</tr>
<tr>
<td>16:0</td>
<td>30.5 ± 16.5</td>
<td>82.8 ± 29.7</td>
<td>52.4 ± 25.4</td>
<td>212.3 ± 157.8**</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>1.8 ± 1.0</td>
<td>9.0 ± 5.3</td>
<td>7.1 ± 5.0</td>
<td>486.8 ± 388.3**</td>
</tr>
<tr>
<td>18:0</td>
<td>16.0 ± 7.8</td>
<td>30.5 ± 7.7</td>
<td>14.5 ± 9.5</td>
<td>120.3 ± 111.3</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>28.5 ± 16.6</td>
<td>82.9 ± 31.3</td>
<td>54.4 ± 28.4</td>
<td>257.1 ± 216.7**</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>24.0 ± 23.5</td>
<td>54.0 ± 28.8</td>
<td>30.1 ± 16.5</td>
<td>222.1 ± 221.0*</td>
</tr>
<tr>
<td>Total</td>
<td>104.3 ± 62.8</td>
<td>267.8 ± 99.0</td>
<td>163.5 ± 84.2</td>
<td>208.4 ± 174.4</td>
</tr>
</tbody>
</table>

*100 × (fasting – nonfasting)/nonfasting. Significance of % increase of individual nonesterified fatty acids vs. 20:4 increase: * P <0.05, ** P <0.01

### References


Direct Amplification of the CAG Repeat of huntingtin Without Amplification of CCG, Cherie E. Bond and M. E. Hodes* (Dept. of Med. and Molec. Genetics, Indiana Univ. School of Med., 975 W. Walnut St., Indianapolis, IN 46202-5251; *author for correspondence: fax 317-274-1069)

Huntington disease is an autosomal dominant, progressive neurodegenerative disorder caused by expansion of a CAG trinucleotide repeat near the 5′ end of the huntingtin gene (HTT) on chromosome 4. Diagnosis of Huntington disease is usually made by a thorough neurological examination after onset of signs. With the advent of molecular DNA analysis, individuals can now be diagnosed long before they present with any signs of the disease.

Unfortunately, there is no clear demarcation of affected vs unaffected status in terms of triplet repeat size. Although a finding of <30 (CAG)n repeats is generally considered to represent a normal phenotype, and >40 repeats generally leads to eventual manifestation of the disease, the large region of overlap between normal and affected alleles complicates predictive testing and diagnosis of individuals, particularly those with no documented family history of Huntington disease.

In addition, most methods currently used to amplify and analyze this area of the genome encompass an adjacent (CCG)n repeat, which also varies in size [2–4]. Inclusion of this adjacent CCG repeat complicates the accurate assessment of CAG repeat length and interferes with genotype determination of those individuals carrying alleles in the intermediate range between normal and expanded sizes. The GC-rich nature of this region has interfered with design of a protocol for amplification of only the CAG repeats. To resolve this problem, most laboratories perform time-consuming multiple PCR reactions with different primer sets or rely on Southern transfer or sequencing to confirm their results. We have developed a compatible primer set and PCR protocol that yields consistent, robust amplification of the CAG-repeat region.

All previously published Huntington disease primer sets were mapped on the known cDNA sequence and analyzed for compatibility in terms of size, empirical melting temperatures (Tm), and pairing homology that could lead to diminished product yield through primer dimerization [1–6]. All the 5′ primers were similar in their region of binding and other variables, so we used one of intermediate length and reasonable Tm, with which we had previously amplified a larger region successfully. The 3′ primer was designed for compatibility and for eliminating both downstream (CCG)n repeat regions. This results in direct amplification of only the variable CAG repeat of interest.

\[
\text{5′-CCTCGAGTCCTCAAGTCCTC-3′, } T_m = 67 ^\circ C \\
\text{3′-GGTGCGCGGTGTTGCTGCTG-3′, } T_m = 73 ^\circ C 
\]

PCR reactions were performed in a volume of 25 μL containing 200 ng of DNA, 2.5 μL of PCR reaction buffer (10× conc.; Boehringer Mannheim, Indianapolis, IN), 35 μL formamide, 150 μL/glycerol, 200 μmol/L dNTPs, 40 ng of HDCB3.1, 100 ng of HD3, 60 ng of γ-32P-end-labeled HDCB3.1, and 0.625 U of Taq DNA polymerase. Addition of 0.5 U of Perfect Match DNA polymerase enhancer (Stratagene, La Jolla, CA), although not necessary, improved product yield, whereas addition of other adjunct reagents had no discernible effect. Product yield was not significantly improved by further optimization of MgCl2 concentration, or by increasing or decreasing the amounts of DNA or primers in the reaction.

Samples were covered with one drop of mineral oil, and PCR was performed in a PE 9600 thermocycler (Perkin-Elmer, Norwalk, CT). After an initial denaturation at 98 °C for 5 min, samples were cycled 40 times (each cycle being 97 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s), followed by a final extension at 72 °C for 10 min. PCR products were separated on a 6% denaturing polyacrylamide gel for about 2.5 h, dried, and used to expose x-ray film (Fig. 1). The fragment sizes were determined by comparison with an M13-40 sequencing ladder, two known controls, and L191F1 [11], a cosmid containing 18 repeats. The number of (CAG)n repeats was calculated as follows: (fragment size – 37)/3.

The initial 55 specimens examined showed consistency with