Clin Chem Lab Med 2007;45(5):565–576 © 2007 by Walter de Gruyter • Berlin • New York. DOI 10.1515/CCLM.2007.110

Review

The role of ethylenediamine tetraacetic acid (EDTA) as in vitro anticoagulant for diagnostic purposes

Giuseppe Banfi[1,3,*], Gian Luca Salvagno[2,3] and Giuseppe Lippi[2,3]

[1] IRCCS Galeazzi and Chair of Clinical Biochemistry, School of Medicine, University of Milan, Milano, Italy
[2] Sezione di Chimica e Microscopia Clinica, Dipartimento di Scienze Morfologico-Biomediche, Università degli Studi di Verona, Verona, Italy
[3] SIBioC (Italian Society of Clinical Biochemistry and Clinical Molecular Biology)–SIMEL (Italian Society of Laboratory Medicine)–CISMEL (Italian Committee for Standardization of Laboratory and Hematological Methods) Inter-associative Study Group on Extra-Analytical Variability in Laboratory Testing

Abstract

Anticoagulants are used to prevent clot formation both in vitro and in vivo. In the specific field of in vitro diagnostics, anticoagulants are commonly added to collection tubes either to maintain blood in the fluid state for hematological testing or to obtain suitable plasma for coagulation and clinical chemistry analyses. Unfortunately, no universal anticoagulant that could be used for evaluation of several laboratory parameters in a sample from a single test tube is available so far. Ethylenediamine tetraacetic acid (EDTA) is a polyprotic acid containing four carboxylic acid groups and two amine groups with lone-pair electrons that chelate calcium and several other metal ions. Calcium is necessary for a wide range of enzyme reactions of the coagulation cascade and its removal irreversibly prevents blood clotting within the collection tube. Historically, EDTA has been recommended as the anticoagulant of choice for hematological testing because it allows the best preservation of cellular components and morphology of blood cells. The remarkable expansion in laboratory test volume and complexity over recent decades has amplified the potential spectrum of applications for this anticoagulant, which can be used to stabilize blood for a variety of traditional and innovative tests. Specific data on the behavior of EDTA as an anticoagulant in hematology, including possible pitfalls, are presented. The use of EDTA for measuring cytokines, protein and peptides, and cardiac markers is described, with an outline of the protection of labile molecules provided by this anticoagulant. The use of EDTA in proteomics and in general clinical chemistry is also described in comparison with other anticoagulants and with serum samples. Finally, the possible uses of alternative anticoagulants instead of EDTA and the potential use of a universal anticoagulant are illustrated.
Clin Chem Lab Med 2007;45:565–76.

Keywords: anticoagulants; blood; laboratory testing; plasma; serum.

*Corresponding author: Prof. Giuseppe Banfi, IRCCS Galeazzi, Via R. Galeazzi 4, 20161 Milano, Italy
Phone: +39-02-66214850, Fax: +39-02-66214806,
E-mail: giuseppebanfi@supereva.it

Introduction

In the field of in vitro diagnostics, anticoagulants are used for preserving whole blood to perform a variety of hematological tests and to obtain plasma for coagulation and clinical chemistry analyses. Heparin is used for clinical chemistry, sodium citrate for coagulation tests, and ethylenediamine tetraacetic acid (EDTA) for hemocytometry. The main property of EDTA, a polyprotic acid containing four carboxylic acid groups and two amine groups with lone pair electrons, is the ability to chelate or complex metal ions in 1:1 metal-EDTA complexes (Figure 1). Owing to its strong complexation with metal ions that are cofactors for enzymes, EDTA is widely used as a sequestering agent to prevent some enzyme reactions from occurring. When blood is collected with no additives within an appropriate container (blood tube), it clots fairly quickly. As calcium ions are necessary for this process, the specific association between the carboxylic groups of EDTA and calcium is a reliable solution to prevent clotting, stabilizing whole blood in a fluid form, as required for some laboratory analyses. EDTA was chosen for hematological tests when aniline-derived dyes were proposed for preparing blood smears from peripheral venous blood. EDTA allows optimal dying with May-Grünwald Giemsa stain. Heparin, conversely, triggers platelet (PLT) activation, is more expensive and affects the staining properties, producing a reddish coloration. Citrate is used as an anticoagulant primarily for coagulation studies. An investigation on the Sysmex CD4000 system (Sysmex Corporation, Hyogo, Japan) indicated that it can be used instead of EDTA for complete blood counting (CBC), using corrections for different dilutions. Blood smears stained using the Wright method were similar to those prepared using EDTA anticoagulated blood (1). Moreover, EDTA showed optimal extended stabilization of blood cells and particles. Owing to these properties, EDTA became the anticoagulant of choice for hematological testing. Three EDTA formulations

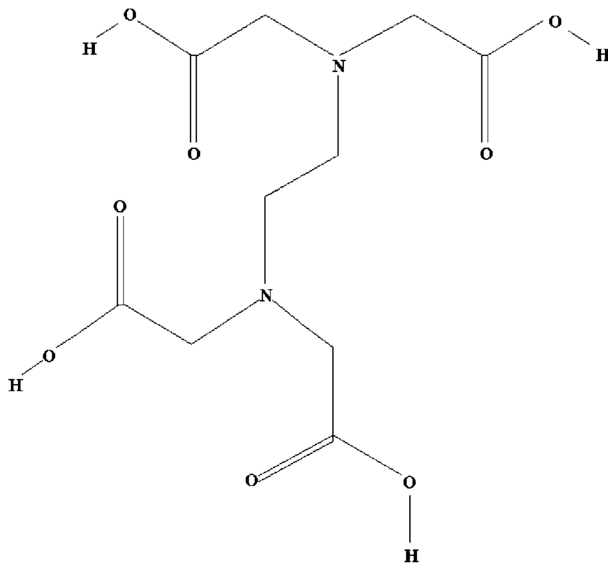

Figure 1 Biochemical structure of ethylenediamine tetra-acetic acid.

are traditionally employed as anticoagulants: Na_2EDTA, K_2EDTA and K_3EDTA, choice of which mostly depends on the type of analyses to be performed. The International Council for Standardization in Hematology (ICSH) currently recommends K_2EDTA as the anticoagulant of choice for hematological testing. This indication has been widely acknowledged in Europe and Japan, whereas K_3EDTA is more commonly used in the US and the UK (2). K_2EDTA is available in a spray-dried form that does not introduce any dilutional effect on small sample volumes and is associated with a less pronounced osmotic effect on blood cells than K_3EDTA (3). An additional advantage of EDTA is that it protects labile molecules; therefore, it may be used to preserve labile analytes, possibly in association with antiproteolytic substances. Nevertheless, the use of EDTA in clinical chemistry is limited owing to its complexation with ions (calcium, iron, magnesium), so that they cannot be measured in EDTA plasma. Moreover, EDTA is not suitable for measuring sodium and potassium since it is used as a sodium or potassium salt. The introduction of innovative automatic analyzers for performing CBC and measuring new erythrocyte and PLT parameters has also raised further complexities and criticisms of the use of EDTA (4).

Hematological testing

EDTA is the anticoagulant of choice for hematological testing. Following the publication of the ISO 6710 procedure, the traditional color code of the stopper for EDTA tubes is lavender. The pH of EDTA varies depending on the salt type: acidity decreases when the number of Na^+ or K^+ ions increases. Free acid solution of EDTA has a pH of 2.5 ± 1.0, whilst the K_3 salt (1% solution) is characterized by a pH of 7.5 ± 1.0. EDTA salts are hyperosmolar, causing water loss from cells. Cell shrinkage is less apparent when K_2 and Na_2EDTA are used. Microhematocrit, the reference ICSH method for packed cell volume (PCV), is not influenced by K_2 or Na_2EDTA, whilst it is decreased by K_3EDTA. Thus, K_2 should be preferred to the K_3 salt, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (5, 6). However, automatic hemocytometers measure the mean corpuscular volume (MCV) and calculate PCV. The effect of cell shrinkage should not be clinically important. In contrast, the concentration is crucial. Dacie and Lewis proposed a K_2EDTA concentration of 1.5 ± 0.25 g/L and a similar concentration is recommended by NCCLS (1.5–2.2 g/L), whereas ICSH recommends a concentration of 4.55 mmol/L of blood (7). When the concentration of EDTA is increased, the MCV measured by automatic instruments is variably influenced, but generally tends to increase (8). This effect is less evident when K_3EDTA is used (8).

Stability of hematological parameters in EDTA-anticoagulated blood

The stability of hematological parameters in blood stored in EDTA is excellent: 48 h for hemoglobin (Hb) and 24 h for red blood cell (RBC) parameters using impedance technologies (9–12). Leukocytes are stable for 24 h when stored at 4°C; the cell differential is also stable in refrigerated samples (13). Reticulocytes (Ret) tend to mature and transform to RBCs in whole blood, but the stability is high (72 h) if EDTA anticoagulated blood is stored at 4°C (14, 15). By this time, however, the indexes mean Hb concentration of Ret (CHCMr) and Ret Hb content (CHr) decrease by 1.2 g/L and 1.8 pg, respectively, whereas the Ret distribution width (RDWr) increases by 2% (16). Therefore, Ret analysis in EDTA samples is recommended within 24 h from drawing (11, 17). The stability of blood specimens for up to 24 h was recently confirmed for the Bayer Advia 120 analyzer. When comparing samples collected in the morning and immediately tested with those stored at 4°C and retested after 24 h, Ret count, Ret%, and CHr differed significantly after 24 h, whereas the immature Ret fraction (IRF) value did not exhibit significant bias. The mean Ret value decreased by 4.8%, with 95% confidence limits of agreement ranging from -20.5% to $+10.8\%$. The difference was statistically significant but clinically negligible (the desirable bias for Ret is $\pm7.8\%$). This study confirmed the reliability of testing most hematological parameters in EDTA samples stored for up to 24 h at 4°C, even in samples from athletes for antidoping or legal purposes (13). The Ret value measured on the Sysmex XT2000 analyzer in EDTA specimens stored at different temperatures for up to 72 h after drawing decreased consistently after 4, 8, 24 and 48 h at room temperature, but was fairly stable in specimens stored at 2°C. The main Ret value was stable for a storage period of up to 72 h, but this was attributed to an artifact of the hematological systems. Although Ret, hematocrit (Ht) and Hb values were substantially stable at 2°C for 24 h, Ht exhibited a significant bias attributed to MCV modifications (18).

Effects of EDTA on platelets

EDTA cannot completely stabilize PLTs, allowing some morphological alterations to occur. When in contact with EDTA, PLTs undergo a time-dependent change from a discoidal to a spherical shape. Although several modern hemocytometers measure the mean PLT volume (MPV), its clinical use has been limited by some preanalytical pitfalls. MPV may be a valid clinical finding for detecting the source of thrombocytopenia. MPV is normal in autoimmune thrombocytopenia, while it is increased in disseminated intravascular coagulation (DIC), microangiopathies and pathologies impairing PLT maturation and release (19). There is a consistent inverse association between the number and volume of PLTs, as demonstrated by Bessman et al. (20); such a relationship is linear up to 400×10^9 PLTs/L. The change in MPV depends on the time of contact with the anticoagulant. This mechanism involves a change in membrane permeability through a cyclic AMP (cAMP)-mediated reaction. The PLT undergoes a spherical change, leading to an apparent increase in volume when the particle passes through an impedance-based analyzer. The volume increase is evident in the 60 min following blood drawing and further stabilizes within 3 h. Therefore, the MPV should be always quantified at a fixed time after blood drawing to allow reliable intra- (longitudinal) and inter-individual comparisons (21). The apparent increase in PLT volume is generally observed using impedance analyzers. In light-scattering-based hemocytometers, for which hematological parameters are defined by both volume and light refraction indexes, the MPV measured is more variable. MPV is usually decreased in these systems, although an increase can be recorded in up to one-third of cases. When the two technologies were compared, there were no significant differences for PLT count, whilst MPV was lower for light scattering-based hemocytometers than for impedance-based instruments. In particular, values recorded by light scattering were higher than those measured by impedance in the 15 min immediately after blood drawing. This has been ascribed to the higher sensitivity of the impedance technology to the initial shape change, whereas light-scatter technologies are influenced afterwards, inducing an apparent decrease in volume (22). Moreover, in some cases the differences between the two technologies are often widened by a type of ''hypersensitivity'' to EDTA, which is accompanied by broad changes in the PLT morphology (22, 23).

Anticoagulants other than EDTA have been proposed for obtaining correct and valid measurements of MPV, including ACD (adenosine, citrate and dextrose) and Na_2EDTA (24), sodium citrate and PGE_1 (prostaglandin E_1) (22), CTAD (citrate, theophylline, adenosine, dextrose) and pyridoxal phosphate, which have been validated on impedance-based systems (21). Citrate alone was considered unreliable for the measurement of MPV, as PLTs slowly adopt a spherical shape, mirroring those changes occurring in EDTA specimens. ACD and a combination of ACD and EDTA were also recommended for the measurement of MPV. In the CTAD mixture, theophylline and dextrose inhibit cAMP phosphodiesterase activity, whereas adenosine stimulates membrane adenylylcyclase. The consequent increase in PLT cAMP and the inhibition of Ca^{2+}-mediated responses lead to a significant decrease in PLT activation (25).

EDTA-induced pseudothrombocytopenia

An additional problem is the potential development of pseudothrombocytopenia in EDTA-anticoagulated specimens, which is typically characterized by a low PLT count due to in vitro PLT clumping or adhesion to white blood cells (WBCs). Owing to the increased volume, these elements are not identified as PLTs by most hemocytometers, but they may be counted as WBCs, producing spurious pseudothrombocytopenia and pseudoleukocytosis diagnoses (26, 27). EDTA-induced pseudothrombocytopenia may be detectable on some automated systems by means of flags and graphics, and can be differentiated by comparing data obtained on EDTA- and sodium citrate-collected specimens. Pseudothrombocytopenia may be associated with negative outcomes if left undetected, triggering unnecessary further investigation, unjustified and invasive pharmacological or medical treatments (28). EDTA-induced pseudothrombocytopenia can be observed in either health or disease and is not related to gender or age. Association with dysfunctions or anomalies of PLT is rare (29). As reported in several epidemiological studies (28, 30–34), the prevalence of EDTA-induced pseudothrombocytopenia is nearly 0.1% and its frequency appears higher in thrombocytopenic patients, ranging from 1.25% to 15.3% (35, 36). Apparently, this form of spurious thrombocytopenia is caused by IgM autoantibodies directed against glycoproteins IIa and IIIb on the PLT surface. In fact, PLTs from patients with Glanzmann disease, which is characterized by a lack of expression of the IIa/IIIb complex, do not react with autoantibodies from pseudothrombocytopenic subjects (37). The IIa/IIIb complex is crucial for PLT adhesion, the first step in aggregation and thrombus formation. EDTA may induce changes in the structural morphology and externalization of the complex, triggering immunological reaction with autoantibodies (27). The phenomenon does not appear to be mediated by calcium chelation and has also been reported for molecules similar to EDTA, such as ethylentriamine pentaacetic acid (38). Moreover, the kinetics is time- but not temperature-dependent (39); it suddenly appears within 2 h after blood drawing (27), as demonstrated by a case during patient hospitalization (40). A sudden onset of EDTA-induced pseudothrombocytopenia was also described following therapy with the glycoprotein IIb/IIIa antagonist c7E3Fab, which was administered to a 63-year-old woman for an emergency coronary intervention (41), and after transcatheter arterial embolization for hepatocellular carcinoma (42). Although the development of EDTA-induced pseudothrombocytopenia basically involves indirect

mechanisms mediated by autoantibodies, a direct causal relationship cannot be ruled out in clinical practice. For example, while EDTA has an inhibitory effect on macrophage adhesion to substrates, displaying a slight anti-inflammatory effect, it is widely applied in endodontic procedures (43). In fact, ^{51}Cr EDTA is a reference method for measuring glomerular filtration rate (44) and EDTA stabilizes polymers used as gastric barriers for some drugs (45). The use of anticoagulants other than EDTA is obviously indicated for identifying this spurious phenomenon and for patient monitoring. In this specific circumstance, reliable anticoagulants are ammonium oxalate (28), heparin (although heparin-induced thrombocytopenia can also occur by a different mechanism) (34), sodium citrate 3.8% v/v (26), and CPT (citrate, pyridoxal phosphate, Tris buffer) (27). The use of aminoglycosides (e.g., kanamycin) is effective in preventing EDTA-induced PLT clumping and dissociation of the clumps in vitro (46). In fact, aminoglycoside supplementation is a reliable means to prevent PLT aggregation when EDTA-anticoagulated samples from EDTA-pseudothrombocytopenic patients are incubated for 30 min at room temperature. It has been reported that kanamycin is also useful for dissociating blood cell aggregates (46).

Effects of EDTA on leukocytes and erythrocytes

Several additional problems associated with the use of EDTA as an anticoagulant have been reported, including leukocyte clumping (47, 48) due to a reaction between IgM autoantibody and neutrophils at room temperature (48). This phenomenon was also reported for lymphoma cells (49). Coincident clumping of WBCs and PLTs has been described at temperatures lower than 37°C; the PLT clumping could not be prevented using anticoagulants other than EDTA (50). In the latter case, two independent mechanisms have been proposed, whereas in other cases IgM paraprotein (51) and IgM cryoagglutinin (52) were involved. Polycarboxylic molecules may induce aspecific RBC agglutination, which can also occur in the presence of EDTA (53). Some difficulties in ABO grouping have been reported, due to either inhibition of antibody antiA1 or activation of an anti-B antibody (54, 55). In some circumstances RBC agglutination simply results from inadequate filling of the primary tube and consequently an improper blood/anticoagulant ratio (56) or on the tube shape [replacement of 55×15 mm EDTA tubes by 84×12 mm tubes was associated with a 2% increased prevalence of PLT clumping (57)]. Although PLT activation in vitro has been described immediately after blood drawing for a variety of anticoagulants, including K_2EDTA, heparin, citrate, and PPACK (D-phenylalanine-L-prolyl-L-argininyl chloroketone), it is particularly apparent using K_2EDTA. Therefore, EDTA and heparin are not recommended for monitoring PLT adhesion (58). A case of pseudoleukocytosis without pseudothrombocytopenia associated with an IgG_2k monoclonal protein and triggered by EDTA has been reported. This phenomenon was also observed using ethylenglycol tetraacetic acid (EGTA) (59). EDTA is generally used for identifying and quantifying leukocyte antigens by flow cytometry (60), although ACD and heparin have been successfully used in some investigations (61).

Cytokines

Cytokines are widely measured for research and clinical purposes. The reliability of cytokine measurement depends on a variety of biological conditions (age, psychophysical stress, exercise, etc.) and is also influenced by several preanalytical variables, including collection and storage procedures for biological samples. EDTA is recommended for cytokine measurement, as it stabilizes the blood until centrifugation. Plasma separation should be completed as quickly as possible; a delay in specimen treatment of more than 1 h induces a decrease of nearly 50% in the original concentration of tumor necrosis factor (TNF) (32, 62, 63). EDTA and cold storage of tubes are hence crucial to maintain cytokine stability in vitro. The most stable cytokine is interleukin 1β (IL-1β) followed by α-interferon (α-IFN), IL-1α, γ-IFN, IL-6, and TNF-α (64). EDTA is not recommended for measuring the soluble receptor of interleukin 2 (sIL-2R) and the transferrin soluble receptor (sTfR). In such cases, a spurious increase has reported compared with serum (65). Sodium citrate has been proposed as the anticoagulant of choice for analysis of TNF-α, because EDTA and heparin may generate spurious increased or decreased concentrations (66), although comparable results were observed for IL-6, TNF-α and leptin between serum and plasma in a separate investigation (67). The concentration of both IL-6 and TNF-α measured in lithium heparin and sodium citrate were significantly lower compared to serum and EDTA plasma, whereas leptin values depended on the anticoagulant used, although the bias did not reach statistical significance (67). The use of whole blood is preferred for studying cytokine induction by bacterial lipopolysaccharide (LPS) because it allows optimal preservation of the physiological milieu in which the interaction between LPS and plasma molecules occurs and leads to cell activation. Heparin enhances LPS-induced production of TNF by monocytes, whilst EDTA inhibits the production of LPS-induced cytokines (68). The phenomenon is associated with the effect of the two anticoagulants on neutrophil-derived protein CAP18 (cationic antimicrobial protein of 18 kDa) and heparin-binding protein (HBP/CAP37), which inhibit and enhance LPS-induced cellular activation, respectively. The inhibitory effect of CAP18 is increased by EDTA and removed by heparin (EDTA abrogates the enhancement of LPS-induced TNF-α production by HBP). Moreover, the LPS-induced TNF-α response is Ca^{2+}-dependent and is inhibited by EDTA, confirming the hypothesis that EDTA is unsuitable for measuring TNF-α (69).

Proteins and peptides

EDTA is the anticoagulant of choice for the collection and storage of molecules characterized by a high

degree of enzymatic degradation in vitro. Such shielding activity may be greatly enhanced by the association of EDTA with aprotinin and other antiproteolytic substances, which inhibit a wide series of physiological proteases (kinins, trypsin, chymotrypsin, and enzymes of leukocytes, coagulation and fibrinolysis) (11). The recommendation to use EDTA for collection and storage of plasma samples for hormone analyses (EDTA plasma stored at 4°C is stable for up to 120 h for most hormones, with the exception of ACTH) has been widely implemented in clinical laboratories (70, 71). In particular, EDTA is recommended for collection and storage of corticotropin (ACTH), parathyroid hormone (PTH), glucagon, C-peptide, vasoactive intestinal peptide (VIP), antidiuretic hormone (ADH), cross-links (CTx), and calcitonin (11). PTH is more stable in EDTA plasma than in serum (72), which is crucial when evaluating samples from hemodialyzed patients (73, 74). Plasma EDTA is also recommended for measuring renin activity. Rapid separation of plasma should be preferred in this circumstance to the use of iced water for tube storage owing to the potential cryoactivation of prorenin (75). Some labile molecules cannot be fully stabilized by EDTA alone: PTH-related peptide (PTH-RP) is stable for 1 h at room temperature and for 24 h at 4°C when using a mix of EDTA, aprotinin, leupeptin and pepstatin (76). EDTA plasma is also preferable for measuring osteocalcin (11); optimal protection of the molecule against the activity of metalloproteases is achieved by increasing the anticoagulant concentration to 5 mmol/L, which also allows two freezing/thawing cycles to be safely performed (77). A special warning should be issued for some test kits containing EDTA in the reaction buffer. Accordingly, some authors recommended the use of serum, possibly supplemented with antiproteolytic substances (78, 79). Procalcitonin (PCT), an innovative and highly specific marker for the diagnosis of clinically relevant bacterial infections and sepsis, should be measured in EDTA plasma stored at room temperature within 4 h of collection. Spurious variations in its concentration have been recorded in both serum (−6.4% and −12.3% at 3 and 24 h) and heparin (+7.6%) (80). An underestimation of α_1-antitrypsin in plasma EDTA has been observed (81). EDTA can also inhibit complement activation; protein protection in some immunoassays was achieved by adding EDTA to the reaction buffer (82), although complete stability of C_{3a}, C_{4a}, C_{5a} is achieved by supplementing EDTA with a protease inhibitor, such as nafamostat mesylate (83, 84). EDTA has been proposed as a reliable alternative for measuring the clotting activity of factor VII (85), whereas it is not recommended for measuring fibrinogen (86). Although total (tPSA) and free prostate-specific antigen (fPSA) are usually measured in serum, EDTA plasma may be also used, as the decay in immunoreactivity at room temperature is less apparent in plasma than in serum (87). A comparison of the stability of different PSA isoforms stored in various media suggests that the rate of decrease in PSA is higher in serum than in EDTA and heparin plasma (88). In a study based on a biological bank where sera and EDTA plasma have been stored for a median time of 20 years, fPSA measurement in serum was judged to be less reliable than in plasma. Therefore, either serum or plasma may be used for measuring tPSA, but plasma should be preferred when fPSA measurement is required. Accordingly, EDTA plasma is recommended for prospective studies and is optimal for retrospective investigations (89). EDTA is not recommended for the analysis of metalloproteases, as it induces a spurious increase in these proteins. Heparin is the anticoagulant of choice in this circumstance (90).

Cardiac markers

In a recent investigation of five different diagnostic kits, the use of EDTA was associated with a variable degree of interference in some myoglobin immunoassays (91). Troponin T (TnT) is underestimated (up to 18%) in EDTA using an immunochemiluminometric assay (92). EDTA can also interfere with TnI activity, as it irreversibly chelates Ca^{2+}, which is necessary for complexation of the molecule. Therefore, TnT and TnI assays are usually performed in serum. Heparin is not indicated for the measurement of troponins. In fact, the negatively charged polyanions on heparin bind to positively charged ions on the troponin molecules, leading to spurious decreases in cTnT and cTnI in plasma heparin. EDTA may also decrease the immunoreactivity of the troponins. EDTA promotes the release of free cTnI from the Ca^{2+}-dependent cTnI-troponin C complex, introducing a negative bias in immunoassays developed with antibodies directed against the complexed TnI (93).

The use of EDTA plasma, with or without antiproteolytic substances, is recommended for the measurement of atrial natriuretic peptide (ANP). The molecule is stable for 1 h at room temperature and 7 h at 4°C. Supplementation of EDTA tubes with phosphoramidone stabilizes ANP for up to 70 h (94). Cold storage of plastic (polypropylene) EDTA tubes is indicated for the quantification of both ANP and brain natriuretic peptide (BNP) (95). Although EDTA plasma and centrifugation of tubes at 4°C is widely recommended for BNP testing (96), heparin plasma may be an alternative (97). The N-terminal fragment of proBNP (NT-proBNP) can be measured in EDTA and heparinized plasma or serum (98–100), although the NT-proBNP concentration in EDTA plasma can be up to 10% lower than in serum or heparin plasma (101). No significant difference could be observed for NT-proBNP or BNP when EDTA anticoagulated blood was aliquoted and stored in glass or plastic tubes for 24 h at room temperature or at 4°C. However, unlike BNP, NT-proBNP is stable in EDTA plasma for 3 days at room temperature or longer at 4°C (100). Accordingly, NT-proBNP, but not BNP, may be stored at −20°C for at least 4 months without a relevant loss of the immunoreactive analyte (102). Standard blood tubes containing EDTA as anticoagulant could be used for the collection of samples for BNP and N-terminal ANP analysis; addition of aprotinin does not improve the

stability of the two peptides compared to EDTA alone (103).

Proteomics

Proteomics has developed through the identification of proteins in serum/plasma by a minimally invasive tool for assessing the presence of disease and monitoring the response to treatment and/or disease progression (104). Although the potential clinical applications of this innovative diagnostic tool are broad-based, recent studies have emphasized the impact of several preanalytical variables on proteomics results, especially for protein patterns of surface-enhanced laser desorption ionization-time of flight (SELDI-TOF) mass spectrometry. The time elapsed from sample collection, for example, influences the protein profile of matrix-assisted laser desorption ionization (MALDI-TOF) mass spectrometry. Protein stabilization by EDTA and antiproteolytic substances would substantially improve the outcome (105, 106). The chelating action of EDTA is advantageous, as the activity of many proteases requires metals. In any case, plasma should be processed within 1 h of specimen collection (105). The influence of sample collection and processing on the spectral patterns of SELDI-TOF mass spectrometry analysis is so critical that the instrument software can even identify the sample source and the day of chip processing and reading and has an accuracy greater than that for cancer detection, which is the ultimate goal of this research (106). Owing to its strong stabilizing effect on several labile molecules, EDTA plasma appears to be a better choice for proteomics studies. Citrate has the advantage of more efficient PLT stabilization, but the plasma dilution is not acceptable. Moreover, heparin binds to a significant number of proteins besides antithrombin and may interfere with the protein profile (107).

Drugs

EDTA is recommended for the measurement of some drugs, such as aminoglycosides. Citrate and heparin may generate spurious decreases in cyclosporin and tacrolimus, which are widely employed immunosuppressants (108, 109). EDTA could also be used as an alternative to heparin or serum for the measurement of several antiepileptic drugs (phenobarbital, phenytoin, ethosuccimide, carbamazepin, and valproate), antiarrhythmic drugs, salicylate, acetaminophen and theophylline (110, 111). Owing to the variable influence of heparin, citrate, oxalate and EDTA, the measurement of free fractions of antiepileptic drugs should be performed on serum (110). Digoxin, but not digitoxin, can be measured in EDTA plasma (111). Finally, EDTA should not be used for the measurement of 3,4-dihydroxyphenylalanine, which undergoes rapid degradation triggered by this anticoagulant (112).

Molecular biology, virology, infectious diseases

EDTA and ACD are the anticoagulants of choice in molecular biology. The use of heparin is not recommended, as it inhibits DNA amplification by polymerase chain reaction (PCR) technology and binds to DNA denaturation products (113, 114). EDTA and ACD may also inhibit restriction enzymes, although they are generally removed during the precipitation steps of this procedure (115). EDTA is recommended for HIV1 RNA quantification by PCR. ACD may be also used, but the plasma must be separated within 6 h of blood drawing. The recovery of HIV1 RNA copies in this period is greater from EDTA plasma than from ACD or heparin plasmas (116). EDTA is recommended for amplification of DNA or RNA in bacteriology and virology (*Mycobacterium* spp., hepatitis B virus, hepatitis C virus (HCV), human T cell leukemia virus type 1 (HTLV1), HIV viral load, human herpes virus 6, JC polyoma virus, measles virus). EDTA stabilizes RNA, the concentration of which may be otherwise increased by release from necrotic and apoptotic blood cells or decreased by degradation from RNases. Plasma RNA is fairly stable in whole blood stored at 4°C; however, whole clotted blood should be stored at 4°C and processed within 6 h to maintain a stable serum RNA concentration (117). Conventional EDTA tubes may be used to collect blood as reliable alternatives to specific plasma preparations and more expensive collection tubes for analyzing HIV1 viral load (118) and for mRNA quantification in real-time PCR (119). HCV-RNA concentrations in EDTA anticoagulated blood are stable for up to 5 days when stored at room temperature (120). The use of special tubes (plasma preparation tubes containing EDTA and gel separating cells and plasma after centrifugation) has little advantage over the storage of whole blood in conventional EDTA tubes in terms of the HCV signal, at least within 72 h from collection. Genomic DNA is released in serum during the clotting process, regardless of the storage conditions; therefore, serum should not be used to monitor the concentration of circulating cell-free DNA when this assay is needed. The bias observed for the measurement of genomic DNA concentration between EDTA plasma (or ACD-derived plasma) and serum does not depend on the inhibitory effect of the anticoagulant, as demonstrated in specific spiking studies (121).

Clinical chemistry

EDTA is traditionally unsuitable for general clinical chemistry analyses owing to its complexation with metal ions. In contrast, heparin may be used to obtain suitable plasma, because anticoagulation is achieved through antithrombin-mediated inhibition of the coagulation factors Xa, IXa and IIa. The use of plasma has been recommended, especially for stat testing. In fact heparin plasma can be obtained much more quickly than serum (12). Nevertheless, EDTA plasma is the anticoagulant of choice for some analyses, such as ammonia and creatine kinase isoforms, for which

chelation of zinc ions is crucial for inhibiting carboxypeptidase (12). EDTA can cause contamination when droplets of the anticoagulant are transferred via a syringe tip from one container to another. This problem is more frequent when using special blood collection systems such as the Monovette (122). The cross-contamination may either induce spurious hyperkalemia or can mask true hypokalemia and hypercalcemia. Although EDTA contamination in the specimen can be detected by dye-binding calcium analysis, this procedure is not recommended as it displays poor sensitivity and is labor-intensive.

EDTA plasma can be used for measuring total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and apolipoproteins. Serum is the biological material conventionally used for these analytes, although several epidemiological investigations on risk factors for heart diseases have been performed on EDTA plasma (123), especially when lipoproteins and apolipoproteins were evaluated (124). A heterogeneous bias is observed when total cholesterol, triglycerides and HDL-C are assayed in EDTA plasma and serum. The concentration of these analytes is underestimated in serum, although the bias depends greatly on the analytical technique (125). Plasma EDTA should be used for homocysteine assays. Although improved stabilization of the molecule can be achieved by 0.5 mol/L citric acid supplementation, which is effective in inhibiting the transformation of methionine and cysteine (126), the concentration of homocysteine in citrate- and fluoride-containing samples is significantly lower than in EDTA plasma (127). The homocysteine concentration is stable for up to 3 h at 0°C in whole blood anticoagulated with EDTA and sodium citrate. Significant overestimation has occasionally been observed when measuring homocysteine in EDTA plasma compared to citrated plasma (128). However, Willems et al. did not confirm this finding in a separate investigation (126), highlighting that the most probable source of this discrepancy may be the use of different analytical techniques (fluorescence or chromatography) (127).

Amino acids, vitamins, and metal ions

Amino acids are actively released from blood cells during the coagulation process, producing spurious increases in serum. Therefore, serum may be unsuitable for measurement of amino acids. Heparin plasma is generally recommended for amino acid assays; however, EDTA plasma should be used for cystine, because the traces of sodium metabisulfite in heparin may interfere in the measurement of sulfated amino acids (129). Plasma EDTA is also recommended for analysis of several vitamins (B_6, B_{12}, A and E) (130), antioxidants (β-carotene and carotenoids) (131), glutathione (supplemented with perchloric acid and phenanthroline) (132) and malondialdehyde (133). Plasma should be separated at 4°C and RBCs should be treated with a 0.5% pyrogallol saline solution when measuring vitamin E in erythrocytes (130). Among trace elements, lead should be measured in EDTA plasma, because heparin produces spurious elevations of this analyte. Serum is also suitable, provided that special tubes with very low metal content are used (134). EDTA plasma is also recommended for cadmium analysis (11). Finally, EDTA-anticoagulated whole blood is commonly used to obtain lysates for the measurement of glycosylated Hb (HbA_{1c}) and for Hb electrophoresis.

New anticoagulants, ''universal'' anticoagulant, and alternatives to EDTA

Regardless of its limitations, EDTA is still the anticoagulant of choice for hematological testing and for particular clinical chemistry analyses. Some promising alternatives have been suggested, with the aim of unifying and limiting the number of collection tubes required for clinical chemistry, hematology and coagulation testing. The use of a ''universal'' anticoagulant would rationalize and optimize laboratory activity, reducing workflow, sample volume and turnaround time. Among others, EDTA has noteworthy potential to fulfill the basic requirements of the ideal anticoagulant, because it can also be used for coagulation testing, even in subjects on oral anticoagulant therapy (135).

Hirudin

Hirudin, a polypeptide of 7 kDa produced from the salivary glands of leeches, inhibits coagulation at very low doses (10 μg per 1 mL of blood) and does not require the presence of antithrombin. Hirudin irreversibly binds by its carboxy-terminal tail to the fibrinogen-binding site of thrombin, inhibiting the conversion of fibrinogen to fibrin. In a recent investigation, results of laboratory testing in samples collected in 4-mL tubes containing a final hirudin concentration of 1000 antithrombotic activity units per mL were compared to those obtained in samples collected in traditional K_2EDTA and serum tubes (136). Overall, the results for CBC and hematological parameters were rather satisfactory. Lower correlation was only recorded for monocytes and basophils; monocytes were occasionally recognized as basophils on the Coulter STKS instrument. Results for serological parameters and routine clinical chemistry testing (24 parameters) did not exhibit significant differences, with the exception of potassium and total protein, the concentrations of which in hirudin plasma were consistently decreased and increased, respectively. The concentration of free Hb in hirudin plasma was also significantly lower than that measured in the corresponding sera. Although single clotting factors such as fibrinogen and antithrombin can be measured in hirudin blood, results for some routine coagulation assays, especially the activated partial thromboplastin time (APTT) and prothrombin time (PT), were unsatisfactory, as hirudinized blood apparently does not clot following the addition of tissue factor and calcium or cephaloplastin. Immunophenotyping and PCR-based methods can be reliably performed in hirudi-

Table 1 Recommendations for use of EDTA in different laboratory analyses.

Analysis	Use of EDTA	Pitfalls and warnings
Hemocytometry	Recommended	Platelet clumping; platelet volume measurement; leukocyte clumping
Cytokines	Recommended	Not recommended for sIL2R, sTfR and TNFα measurement
Proteins and peptides	Recommended for molecules characterized by a high degree of enzymatic degradation in vitro	
Cardiac markers	Recommended for natriuretic peptide measurement	Not recommended for troponin measurement
Proteomics	Recommended	
Drugs	Recommended for aminoglycosides and antiepileptic drugs	
Molecular biology	Recommended	
Virology	Recommended	
Clinical chemistry	Recommended only for ammonia, CK isoforms and HbA_{1c}	Not generally recommended

nized blood. Carboxy-terminal portions of the molecule that have anticoagulant properties have recently been isolated (hirulogs) (137); however, standard hirudinized tubes have not been produced or commercialized so far.

Synthetic thrombin inhibitors

Some other synthetic thrombin inhibitors have been produced, such as argatroban (138) and DX-9065a (139). These compounds may be suitable for hematological testing, although significant differences were observed for serum in the measurement of potassium, total protein and cholinesterase. PPACK is a thrombin inhibitor used for clinical chemistry and Ht testing in point-of-care instruments (140). Argatroban can be used for the same purposes, but higher concentrations are needed (245 vs. 75 μmol/L for PPACK) (141). Sulfonated polyisoprene (SPIP) is another excellent new anticoagulant that can be used for evaluation of several laboratory parameters within a single test tube in routine laboratory work. However, several improvements are required for maintaining the reliability of hematological, biochemical and electrolyte measurements (142).

Conclusions

The addition of anticoagulants and preservatives to blood collection tubes is almost unavoidable to preserve blood or obtain suitable plasma for in vitro testing, but it influences the composition of the specimen and may interfere with some analyses. Accordingly, certain anticoagulants are better suited for specific determinations, whereas other may be contraindicated. The search for an ideal anticoagulant has spanned decades and several approaches have been used to identify novel molecules. Unfortunately, a universal anticoagulant for laboratory testing is still elusive. Nevertheless, this is a promising area of research for the diagnostics industry. The introduction of a universal anticoagulant would yield enormous potential to rationalize the entire laboratory workflow, possibly eliminating preanalytical bias in serum and whole blood, reducing the number of error-prone steps such as collection, handling and sorting of separate blood tubes, and ultimately improving the time- and cost-effectiveness of the total testing process. EDTA is an optimal anticoagulant, which also serves as a stabilizing agent for labile molecules. Besides its broad use in laboratory hematology, further perspectives are emerging for this classical anticoagulant, including blood collection and storage for proteomics, molecular biology, microbiology, drug monitoring, clinical chemistry and coagulation testing, as summarized in Table 1.

References

1. Perrotta G, Roberts L, Glazier J, Schumacher HR. Use of sodium citrate anticoagulant for routine haematological analysis on the CD4000: an opportunity to enhance efficiency in the clinical laboratory. Lab Hematol 1998;4:156–62.
2. Okada T. Development and problems of automated hematology analyzers. Sysmex J Int 1999;9:52–7.
3. International Council for Standardization in Haematology. Recommendations of the International Council for Standardization in Haematology for ethylenediamine tetraacetic acid anticoagulation of blood for blood cell counting and sizing: expert panel on cytometry. Am J Clin Pathol 1993;100:371–2.
4. Reardon DM, Warner B, Trowbridge EA. EDTA, the traditional anticoagulant of haematology: with increased automation is it time for review? Med Lab Sci 1991;48:72–5.
5. Narayanan S. Effect of anticoagulants used for blood collection on laboratory tests. Proc JCLA 1993;7:1–10.
6. National Committee for Clinical Laboratory Standards. H35-P. Additives to blood collection devices: EDTA. Proposed standard. Villanova, PA: NCCLS, 1989;9:851–74.
7. Dacie JV, Lewis SM. Practical haematology, 6th ed. Edinburgh: Churchill Livingstone, 1984:3.
8. Goossens W, Van Duppen V, Verwilghen RL. K_2 or K_3EDTA: the anticoagulant of choice in routine haematology? Clin Lab Haematol 1991;13:291–5.

9. Felding P, Hyltoft Petersen P, Hørder M. The stability of blood, plasma and serum constituents during simulated transport. Scand J Clin Lab Invest 1981;41:35–40.
10. Kouri T, Siloaho M, Pohjavaara S, Koskinen P, Malminiemi O, Pohja-Nylander P, et al. Preanalytical factors and measurement uncertainty. Scand J Clin Invest 2005;65:463–76.
11. Guder WG, Ehret W, Fonseca-Wollheim F, Heil W, Muller-Plathe O, Schmitt Y, et al. The quality of diagnostic samples. Lab Med 2002;26:267–83.
12. Forum of European Societies of Clinical Chemistry (FESCC). The quality of diagnostic samples. Available at: http://www.diagnosticsample.com/. Accessed December 8, 2006.
13. Lippi G, Salvagno GL, Solero GP, Franchini M, Guidi GC. Stability of blood cell counts, hematologic parameters and reticulocytes indexes on the Advia A120 hematologic analyzer. J Lab Clin Med 2005;146:333–40.
14. National Committee for Clinical Laboratory Standard. Methods for reticulocyte counting (flow cytometry and supravital dyes); approved guideline. H44-A. Villanova, PA: NCCLS, 1997.
15. Cavill I, Kraaijenhagen R, Pradella R, D'Onofrio G, Herkner K, Rowan RM, et al. In vitro stability of the reticulocyte count. Clin Lab Haematol 1996;18:9–11.
16. Brugnara C, Colella GM, Cremins JC, Langley RC, Schneider TJ, Rutherford CR, et al. Effects of subcutaneous recombinant human erythropoietin in normal subjects: development of decreased reticulocyte hemoglobin content and iron-deficient erythropoiesis. J Lab Clin Med 1994;123:660–7.
17. Banfi G, Dolci A. Preanalytical phase of sport biochemistry and haematology. J Sports Med Phys Fitness 2003;43:223–30.
18. Robinson N, Mangin P, Saugy M. Time and temperature dependant changes in red blood cell analytes used for testing recombinant erythropoietin abuse in sports. Clin Lab 2004;50:317–23.
19. Karpatkin S, Freedman ML. Hypersplenic thrombocytopenia differentiated from increased destruction by platelet volume. Ann Int Med 1978;89:200–3.
20. Bessman JD, Williams LJ, Gilmer PR. Platelet size in health and hematologic disease. Am J Clin Pathol 1982;78:150–3.
21. Lippi U, Schinella M, Modena M, Nicoli M. A new anticoagulant solution to stabilize platelet indices. Prog Med Lab 1989;3:111–5.
22. Trowbridge EA, Reardon DM, Hutchinson D, Pickering C. The routine measurement of platelet volume: a comparison of light scattering and aperture-impedance technologies. Clin Phys Physiol Meas 1985;6:221–38.
23. Reardon D, Hutchinson D, Bradley L, Trowbridge EA. Automated haematology: a comparative study of cell counting and sizing using aperture impedance and flow cytometric systems. Med Lab Sci 1987;44:320–5.
24. Thompson CB, Diaz DD, Quinn PG, Lapins M, Kurtz SR, Valeri CR. The role of anticoagulation in the measurement of platelet volumes. Am J Clin Pathol 1983;80: 327–32.
25. Macey M, Azam U, McCarthy D, Webb L, Chapman ES, Okrongly D, et al. Evaluation of the anticoagulants EDTA and citrate, theophylline, adenosine, and dipyridamole (CTAD) for assessing platelet activation on the ADVIA 120 hematology system. Clin Chem 2002;48:891–9.
26. Banfi G. La pseudotrombocitopenia da EDTA. Biochim Clin 1987;11:957–62.
27. Lippi U, Schinella M, Biasioli B. Le pseudotrombocitopenie. Prog Med Lab 1991;5:81–8.
28. Payne BA, Pierre RV. Pseudothrombocytopenia: a laboratory artifact with potentially serious consequences. Mayo Clin Proc 1984;59:123–5.
29. Silvestri F, Masotti A, Pradella P, Zaja F, Barillari G, De Marco L. More on false thrombocytopenias: EDTA-dependent pseudothrombocytopenia associated with a congenital platelet release defect. Vox Sang 1996;71: 27–9.
30. Savage RA. Pseudoleukocytosis due to EDTA-induced platelet clumping. Am J Clin Pathol 1984;81:317–22.
31. Mant MJ, Doery JC, Gauldie J, Sims H. Pseudothrombocytopenia due to platelet aggregation and degranulation in blood collected in EDTA. Scand J Hematol 1975;15:161–70.
32. Bartels PC, Schoorl M, Lombarts AJ. Screening for EDTA-dependent deviations in platelet counts and abnormalities in platelet distribution histograms in pseudothrombocytopenia. Scand J Clin Lab Invest 1997;57:629–36.
33. Vicari A, Banfi G, Bonini PA. EDTA-dependent pseudothrombocytopaenia: a 12-month epidemiological study. Scand J Clin Lab Invest 1988;48:537–42.
34. Mant MJ, Doery JC, Gauldie J, Sims H. Pseudothrombocytopenia due to platelet aggregation and degranulation in blood collected in EDTA. Scand J Hematol 1975;15:161–70.
35. Manthorpe R, Kofod B, Wiik A, Svehag SE. Pseudothrombocytopenia. In vitro studies on the underlying mechanism. Scand J Hematol 1981;26:385–90.
36. Silvestri F, Virgolini L, Savignano C, Zaja F, Velisig M, Baccarani M. Incidence and diagnosis of EDTA-dependent pseudothrombocytopenia in a consecutive outpatient population referred for isolated thrombocytopenia. Vox Sang 1995;68:35–9.
37. Pegels JG, Bruynes EC, Engelfriet CP, von dem Borne EG. Pseudothrombocytopenia: an immunologic study on platelet antibodies dependent on ethylene diamine tetraacetate. Blood 1982;59:157–61.
38. Onder O, Weinstein A, Hoyer LW. Pseudothrombocytopenia caused by platelet agglutinins that are reactive in blood anticoagulated with chelating agents. Blood 1980;56:1977–82.
39. Veenhoven WA, van der Schans GS, Huiges W, Metting-Scherpuis HE, Halie MR, Nieweg HO. Pseudothrombocytopenia due to agglutinins. Am J Clin Pathol 1979; 72:1005–8.
40. Gschwandtner ME, Siostrzonek P, Bodinger C, Neunteufl T, Zauner C, Heinz G, et al. Documented sudden onset of pseudothrombocytopenia. Ann Hematol 1997;74: 283–5.
41. Stiegler H, Fischer Y, Steiner S, Strauer BE, Reinauer H. Sudden onset of EDTA-dependent pseudothrombocytopenia after therapy with the glycoprotein IIb/IIIa antagonist c7E3 Fab. Ann Hematol 2000;79:161–4.
42. Yoshikawa T, Nakanishi K, Maruta T, Takenaka D, Hirota S, Matsumoto S, et al. Anticoagulant-induced pseudothrombocytopenia occurring after transcatheter arterial embolization for hepatocellular carcinoma. Jpn J Clin Oncol 2006;36:527–31.
43. Segura JJ, Calvo JR, Guerrero JM, Jimenez-Planas A, Sampedro C, Llamas R. EDTA inhibits in vitro substrate adherence capacity of macrophages: endodontic implications. J Endod 1997;23:205–8.
44. Grubb A, Nordin G. Notable steps in obtaining improved estimates for glomerular filtration rate. Clin Chem 2006;52:169–70.
45. Bernkop-Schnurch A, Scerbe-Saiko A. Synthesis and in vitro evaluation of chitosan-EDTA-protease inhibitor conjugates which might be useful in oral delivery of peptides and proteins. Pharm Res 1998;15:263–9.
46. Sakurai S, Shiojima I, Tanigawa T, Nakahara K. Aminoglycosides prevent and dissociate the aggregation of platelets in patients with EDTA-dependent pseudothrombocytopenia. Br J Haematol 1997;99:817–23.

47. Epstein HD, Kruskall MS. Spurious leukopenia due to in vitro granulocyte aggregation. Am J Clin Pathol 1988;89:652–5.
48. Hillyer CD, Knopf AN, Berkman EM. EDTA-dependent leukoagglutination. Am J Clin Pathol 1990;94:458–61.
49. Juneja S, Wolf M, McLennan R. Clumping of lymphoma cells in peripheral blood induced by EDTA. J Clin Pathol 1992;45:538–40.
50. Moraglio D, Banfi G, Arnelli A. Association of pseudothrombocytopenia and pseudoleukopenia: evidence for different pathogenic mechanisms. Scand J Clin Lab Invest 1994;54:257–65.
51. Pujol M, Ribera JM, Jimenez C, Ribera A, Abad E, Feliu E. Essential monoclonal gammopathy with an IgM paraprotein that is a cryoglobulin with cold agglutinin and EDTA-dependent platelet antibody properties. Br J Haematol 1998;100:603–4.
52. De Caterina M, Fratellanza G, Grimaldi E. Evidence of a cold immunoglobulin M autoantibody against 78-kD platelet glycoprotein in a case of EDTA-dependent pseudothrombocytopenia. Am J Clin Pathol 1993;99:163–7.
53. Beck ML, Freihaut B, Henry R, Pierce S, Bayer WL. A serum hemagglutinating property dependent upon polycarboxylic groups. Br J Hematol 1975;29:145–56.
54. Tedrow HE, Ziegler ZR. Evidence of anti-A1 inhibited by EDTA. Transfusion 1988;28:177–8.
55. Yasuda H, Ohto H, Motoki R, Uchikawa M. An EDTA-associated anti-B agglutinin: the role of ionized calcium. Transfusion 1997;37:1131–6.
56. Pewarchuk W, VanderBoom J, Blajchman MA. Pseudopolycythemia, pseudothrombocytopenia, and pseudoleukopenia due to overfilling of blood collection vacuum tubes. Arch Pathol Lab Med 1992;116:90–2.
57. Davis W. Abnormal platelet aggregation in elongated sample bottles. Clin Lab Haematol 1993;15:231–7.
58. Golanski J, Pietrucha T, Baj Z, Greger J, Watala C. Molecular insights into the anticoagulant-induced spontaneous activation of platelets in whole blood – various anticoagulants are not equal. Thromb Res 1996;83:199–216.
59. Shimasaki AK, Fujita K, Fujio S, Sakurabayashi I. Pseudoleukocytosis without pseudothrombocytopenia induced by the interaction of EDTA and IgG_2k M-protein. Clin Chim Acta 2000;299:119–28.
60. Macey MG, McCarthy DA, Davies C, Newland AC. The Q-Prep system: effects on the apparent expression of leucocyte cell surface antigens. Cytometry 1997;30:67–71.
61. Carter PH, Resto Ruiz S, Washington GC, Ethridge S, Palini A, Vogt R, et al. Flow cytometric analysis of whole blood lysis, three anticoagulants, and five cell preparations. Cytometry 1992;13:68–74.
62. Exley A, Cohen J. Optimal collection of blood samples for the measurement of tumor necrosis factor alpha. Cytokine 1990;2:353–6.
63. Riches P, Gooding R, Millar B, Rowbottom A. Influence of collection and separation of blood samples on plasma IL-1, IL-6 and TNFalpha concentrations. J Immunol Methods 1992;153:125–7.
64. Dugué B, Leppänen E, Gräsbeck R. Preanalytical factors and the measurement of cytokines in human subjects. Int J Clin Lab Res 1996;26:99–105.
65. De Jongh R. The influence of anticoagulation and timing of centrifugation on the measurement of blood proteins. In: Proceedings of the 2nd Symposium on Impact of the Preanalytical Phase, Oxford, UK, Jul 5–7, 1996:31–2.
66. Hoffmann JN, Hartl WH, Faist E, Jochum M, Inthron D. Tumor necrosis factor measurement and use of different anticoagulants: possible interference in plasma samples and supernatants from endotoxin-stimulated monocytes. Inflamm Res 1997;46:342–7.
67. Flower L, Ahuja RH, Humphries SE, Mohamed-Ali V. Effects of sample handling on the stability of interleukin 6, tumor necrosis factor alpha and leptin. Cytokine 2000;12:1712–6.
68. Engstad CS, Guttenberg TJ, Osterud B. Modulation of blood cell activation by four commonly used anticoagulants. Thromb Haemost 1997;77:690–6.
69. Brunialti MK, Kallas EG, Freudenberg M, Galanos C, Salomao R. Influence of EDTA and heparin on lipopolysaccharide binding and cell activation, evaluated at single-cell level in whole blood. Cytometry 2002;50:14–8.
70. Banfi G. State of art of preanalysis in laboratories in Italy performing endocrinological tests. Eur J Clin Chem Clin Biochem 1995;33:99–101.
71. Evans MJ, Livesey JH, Jane Ellis M, Yandle TG. Effect of anticoagulants and storage temperatures on stability of plasma and serum hormones. Clin Biochem 2001;34:107–12.
72. Glendenning P, Laffer LL, Weber HK, Musk AA, Vasikaran SD. Parathyroid hormone is more stable in EDTA plasma than in serum. Clin Chem 2002;48:766–7.
73. Teal TK, Wood JL, Stevens PE, Lamb EJ. Stability of bio-intact (1–84) parathyroid hormone ex vivo in serum and EDTA plasma from hemodialysis patients. Clin Chem 2004;50:1713–4
74. Twomey PJ, Whitlock T, Pledger DR. Differences between serum and plasma for intact parathyroid hormone measurement in patients with chronic renal failure in routine clinical practice. J Clin Pathol 2005;58:1000–1.
75. Sealey JE. Plasma renin activity and plasma prorenin assays. Clin Chem 1991;37:1811–9.
76. Pandian MR, Morgan CH, Carlton E, Segre GV. Modified immunoradiometric assay of parathyroid hormone-related protein: clinical application in the differential diagnosis of hypercalcemia. Clin Chem 1992;38:282– 8.
77. Durham BH, Robinson J, Fraser WD. Differences in the stability of intact osteocalcin in serum, lithium heparin plasma and EDTA plasma. Ann Clin Biochem 1995;32:422–3.
78. Banfi G, Daverio R. In vitro stability of osteocalcin. Clin Chem 1994;40:833–4.
79. Power MJ, O'Dwyer B, Breen E, Fottrell PF. Osteocalcin concentrations in plasma prepared with different anticoagulants. Clin Chem 1991;37:281–4.
80. Meisner M, Tschaikowsky K, Schnabel S, Schmidt J, Katalinic A, Schuttler J. Procalcitonin – influence of temperature, storage, anticoagulation and arterial or venous asservation of blood samples on procalcitonin concentrations. Eur J Clin Chem Clin Biochem 1997;35:597–601.
81. Berninger RW, Teixeira MF. α_1-Antitrypsin: the effect of anticoagulants on the trypsin inhibitory capacity, concentration and phenotype. J Clin Chem Clin Biochem 1985;23:277–81.
82. Butler J. Negative interference in immunoassays. Clin Chem 1995;41:481–2.
83. Watkins J, Wild G, Smith S. Nafamostat to stabilize plasma samples taken for complement measurements. Lancet 1989;1:896–7.
84. Pfeifer PH, Kawahara MS, Hugli TE. Possible mechanism for in vitro complement activation in blood and plasma samples: futhan/EDTA controls in vitro complement activation. Clin Chem 1999;45:1190–9.
85. Bladbjerg EM, Overgaard K, Gram J, Jespersen J. The protein concentration of blood coagulation factor VII can be measured equally well in plasma and serum. Scand J Clin Lab Invest 1995;55:267–71.
86. Gleeson M, Kinlay S. Choice of anticoagulants affects measurement of plasma fibrinogen. Clin Chem 1993;39:1754–5.

87. Banfi G. Präanalytik des prostata-spezifischen antigens. Lab Med 1998;22:87–9.
88. Piironen T, Pettersson K, Suonpaa M, Stenman UH, Oesterling JE, Lovgren T, et al. In vitro stability of free pros-
tate specific antigen (PSA) and prostate specific antigen complexed to α_1-antichymotrypsin in blood samples. Urology 1996;48:81–7.
89. Ulmert D, Becker C, Nilsson JA, Piironen T, Bjork T, Hugosson J, et al. Reproducibility and accuracy of measurements of free and total prostate-specific antigen in serum vs. plasma after long-term storage at −20°C. Clin Chem 2006;52:235–9.
90. Jung K, Laube C, Lein M, Lichtinghagen R, Tscheche H, Schnorr D, et al. Kind of sample as preanalytical determinant of matrix metalloproteinase 2 and 9 and tissue inhibitor of metalloproteinase 2 in blood. Clin Chem 1998;44:1060–2.
91. Panteghini M, Altinier S, Dolci A, Pagani F, Pergolini P, Vernocchi A, et al. Multicenter evaluation of five methods for myoglobin determination. Clin Chem 1998;44: A121.
92. Dolci A. Preanalytical phase of cardiac markers. In: Proceedings of the 4th Expert Symposium ''Biological and technical aspects of the preanalytical phase in clinical chemistry and laboratory medicine'' Karlovy Vary, Czech Republic, Sept. 24–25, 1998.
93. Gerhardt W, Nordin G, Herbert AK, Burzell BL, Isaksson A, Gustavsson E, et al. Troponin T and I assays show decreases concentrations in heparin plasma compared with serum: lower recoveries in early than in late phases of myocardial injury. Clin Chem 2000;46:817–21.
94. Artner-Dworzak E, Lindner H, Puschendorf B. In vitro stability of human natriuretic peptide (h-ANP). Clin Chim Acta 1991;203:235–42.
95. Clerico A, Iervasi G, Del Chicca MG, Emdin M, Maffei S, Nannipieri M, et al. Circulating levels of cardiac natriuretic peptides (ANP and BNP) measured by highly sensitive and specific immunoradiometric assays in normal subjects and in patients with different degrees of heart failure. J Endocrinol Invest 1998;21:170–9.
96. Tsuji T, Imagawa K, Masuda H, Harikawa M, Shibata K, Kono M, et al. Stabilization of human brain natriuretic peptide in blood samples. Clin Chem 1994;40:672–3.
97. Dupuy AM, Terrier N, Dubois M, Boularan AM, Cristol JP. Heparin plasma sampling as an alternative to EDTA for BNP determination on the Access-Beckman Coulter – effect of storage at −20°C. Clin Lab 2006;52:393–7.
98. Numata Y, Dohi K, Furukawa A, Kikuoka S, Asada H, Fukunaga T, et al. Immunoradiometric assay for the N-terminal fragment of proatrial natriuretic peptide in human plasma. Clin Chem 1998;44:1008–13.
99. Lippi G, Salvagno GL, Montagnana M, Schena F, Ballestrieri F, Guidi GC. Influence of physical exercise and relationship with biochemical variables of NT-pro-brain natriuretic peptide and ischemia modified albumin. Clin Chim Acta 2006;367:175–80.
100. Yeo KT, Wu AH, Apple FS, Kroll MH, Christenson RH, Lewandrowski KB, et al. Multicenter evaluation of the Roche NT-proBNP assay and comparison to the Biosite Triage BNP assay. Clin Chim Acta 2003;338:107–15.
101. Sokoll LJ, Baum H, Collinson PO, Gurr E, Haass M, Luthe H, et al. Multicenter analytical performance evaluation of the Elecsys proBNP assay. Clin Chem Lab Med 2004;42:965–72.
102. Mueller T, Gegenhuber A, Dieplinger B, Poelz W, Haltmayer M. Long-term stability of endogenous B-type natriuretic peptide (BNP) and amino-terminal proBNP (NT-proBNP) in frozen plasma samples. Clin Chem Lab Med 2004;42:942–4.
103. Buckley MG, Marcus NJ, Yacoub MH. Cardiac peptide stability, aprotinin and room temperature: importance for assessing cardiac function in clinical practice. Clin Sci 1999;97:689–95.
104. Plebani M. Proteomics: the next revolution in laboratory medicine? Clin Chim Acta 2005;357:113–22.
105. Banks RE, Stanley AJ, Cairns DA, Barrett JH, Clarke P, Thompson D, et al. Influences of blood sample processing on low-molecular-weight proteome identified by surface-enhanced laser desorption/ionization mass spectrometry. Clin Chem 2005;51:1637–49.
106. Karsan A, Eigl BJ, Flibotte S. Analytical and preanalytical biases in serum proteomic pattern analysis for breast cancer diagnosis. Clin Chem 2005;51:1525–8.
107. Rai AJ, Vitzthum F. Effects of preanalytical variables on peptide and protein measurements in human serum and plasma: implications for clinical proteomics. Expert Rev Proteomics 2006;3:409–26.
108. Rodriguez-Mendizabal M, Lucena MI, Cabello MR, Blanco E, Lopez-Rodriguez B, Sanchez de la Cuesta F. Variations in blood levels of aminoglycosides related to in vitro anticoagulant usage. Ther Drug Monit 1998;20: 88–91.
109. Shaw LM, Bowers L, Demers L, Freeman D, Moyer T, Sanghvi A, et al. Critical issues in cyclosporine monitoring: report of the task force on cyclosporine monitoring. Clin Chem 1987;33:1269–88.
110. Warner A, Privitera M, Bates D. Standards of laboratory practice: antiepileptic drug monitoring. Clin Chem 1998;44:1085–95.
111. Valdes R Jr, Jortani SA, Gheorghiade M. Standards of laboratory practice: cardiac drug monitoring. Clin Chem 1998;44:1096–109.
112. Eriksson T, Holmgren B. Rapid degradation of 3,4-dihydroxyphenylalanine in plasma containing EDTA. Clin Chim Acta 1995;238:209–16.
113. Narayanan S. Preanalytical and analytical pitfalls in molecular biology techniques. J Clin Ligand Assay 1997;20:200–5.
114. Todd DM, Buccini FJ. Apparent heparin interference with restriction enzyme digestion of genomic DNA. Clin Chem 1993;39:362–3.
115. Coad JE, Lander TA, Litz CE. Inhibition of restriction endonucleases by common clinical anticoagulants. Anal Biochem 1992;205:368–9.
116. Dickover RE, Herman SA, Saddiq K, Wafer D, Dillon M, Bryson YJ. Optimization of specimen-handling procedures for accurate quantitation of levels of human immunodeficiency virus RNA in plasma by reverse transcriptase PCR. J Clin Microbiol 1998;36:1070–3.
117. Tsui NB, Ng EK, Lo YM. Stability of endogenous and added RNA in blood specimens, serum and plasma. Clin Chem 2002;48:1647–53.
118. Giordano M, Kelleher T, Colonno RJ, Lazzarin A, Squires K. The effects of the Roche Amplicor HIV1 Monitor ultrasensitive test versions 1.0 and 1.5 viral load assays and plasma collection tube type on determination of response to antiretroviral therapy and the inappropriateness of cross-study comparisons. J Clin Virol 2006;35:420–5.
119. Isaksson HS, Nilsson TK. Preanalytical aspects of quantification TaqMan real time RT-PCR: applications for TF and VEGF mRNA quantification. Clin Biochem 2006; 39:373–7.
120. Grant PR, Kitchen A, Barbara JA, Hewitt P, Sims CM, Garson JA, et al. Effects of handling and storage of blood on the stability of hepatitis C virus RNA: implications for NAT testing in transfusion practice. Vox Sang 2000;78:137–42
121. Lee TH, Montalvo L, Chrebtow V, Busch MP. Quantification of genomic DNA in plasma and serum samples: higher concentration of genomic DNA found in serum than in plasma. Transfusion 2001;41:276–82.

122. Davidson DF. Effects of contamination of blood specimens with liquid potassium-EDTA anticoagulant. Ann Clin Biochem 2002;39:273–80.
123. Lipid Research Clinics Program. The Lipid Research Clinics Coronary Primary Prevention Trial Results: I. Reduction in incidence of coronary heart disease. J Am Med Assoc 1984;251:351–64.
124. Cooper GR, Myers GL, Smith SJ, Sampson EJ. Standardization of lipid, lipoprotein, and apolipoprotein measurements. Clin Chem 1988;34:B95–105.
125. Beheshti I, Wessels LM, Eckfeldt JH. EDTA-plasma vs. serum differences in cholesterol, high-density-lipoprotein cholesterol, and triglyceride as measured by several methods. Clin Chem 1994;40:2088–92.
126. Willems HP, Bos GM, Gerrits WB, den Heijer M, Vloet S, Blom HJ. Acidic citrate stabilizes blood samples for assays of total homocysteine. Clin Chem 1998;44:342–5.
127. Caliskan S, Kuralay F, Onvural B. Effects of anticoagulants on plasma homocysteine determination. Clin Chim Acta 2001;309:53–6.
128. Salazar JF, Herbeth B, Siest G, Leroy R. Stability of blood homocysteine and other thiols: EDTA or acidic citrate. Clin Chem 1999;45:2016–9.
129. Chuang CK, Lin SP, Lin YT, Huang FY. Effects of anticoagulants in amino acid analysis: comparison of heparin, EDTA, and sodium citrate in Vacutainer tubes for plasma preparation. Clin Chem 1998;44:1052–6.
130. Simon E, Paul JL, Soni T, Simon A, Moatti N. Plasma and erythrocyte vitamin E content in asymptomatic hypercholesterolemic subjects. Clin Chem 1997;43:285–9.
131. Gross MD, Prouty CB, Jacobs DR Jr. Stability of carotenoids and α-tocopherol during blood collection and processing procedures. Clin Chem 1995;41:943–4.
132. Dufaux B, Heine O, Kothe A, Prinz U, Rost R. Blood glutathione status following distance running. Int J Sports Med 1997;18:89–93.
133. Nielsen F, Mikkelsen BB, Nielsen JB, Andersen HR, Grandjean P. Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors. Clin Chem 1997;43:1209–14.
134. Smith DR, Ilustre RP, Osterloh JD. Methodological consideration for the accurate determination of lead in human plasma and serum. Am J Ind Med 1998;33:430–8.
135. Horsti J. Use of EDTA samples for prothrombin time measurement in patients receiving oral anticoagulants. Haematologica 2001;86:851–5.
136. Menssen HD, Brandt N, Leben R, Muller F, Thiel E, Melber K. Measurement of haematological, clinical chemistry, and infection parameters from hirudinized blood collected in universal blood sampling tubes. Semin Thromb Hemost 2001;27:349–56.
137. Maraganore JM, Bourdon P, Jablonski J, Ramachandran KL, Fenton JW. Design and characterization of hirulogs: a novel class of bivalent peptide inhibitors of thrombin. Biochemistry 1990;29:7095–101.
138. Matsuo T, Kario K, Kodama K, Okamoto S. Clinical application of the synthetic thrombin inhibitor Argatroban (MD805). Semin Thromb Hemost 1992;18:155–60.
139. Kumura T, Hino M, Yamane T, Tominaga K, Tatsumi N. DX-9065a, a specific factor Xa inhibitor, as a universal anticoagulant for blood collection tubes. Clin Chim Acta 2000;294:27–35.
140. Lyon ME, Fine JS, Henderson PJ, Lyon AW. PPACK: alternative anticoagulant to heparin salts for blood gas and electrolyte specimens. Clin Chem 1995;41:1038–41.
141. Lyon AW, Harding SR, Drobot D, Lyon ME. Use of thrombin inhibitors ex vivo allows critical care clinical chemistry and hematology testing on common specimens. Clin Biochem 1997;30:121–7.
142. Kinoshita Y, Ohta K, Yamane T, Hino M, Takubo T, Samori T, et al. Synthetic polymer sulphonated polyisoprene as a universal anticoagulant for laboratory testing. J Clin Lab Anal 2000;14:180–7.

Received December 11, 2006, accepted January 25, 2007