

The Use of Hirudin as Universal Anticoagulant in Haematology, Clinical Chemistry and Blood Grouping

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Undesirable interactions between anticoagulants and diagnostic test kit procedures so far have prevented the development of a single uniform blood sampling tube. Contrary to K₂-EDTA, heparin and other anticoagulants, hirudin only minimally alters blood cells and dissolved blood constituents, thus qualifying as a universal anticoagulant for diagnostic purposes. Automated complete blood counts, automated analyses of clinical chemistry analytes and immunohaematology were performed from hirudinised and routinely processed blood obtained from healthy volunteers (n=35) and hospitalised patients (n=45). Hirudin (400 ATU/ml blood) sufficiently anticoagulated blood for diagnostic purposes. The measurements of automated complete blood counts obtained from K₂-EDTA-anticoagulated and hirudinised blood correlated significantly as did the measurements of 24 clinical chemistry analytes from hirudinised plasma and serum. Regression analysis revealed that the results of complete blood counts and clinical chemistry tests were predictable from the respective measurements from hirudinised blood (p=0.001). Immunohaematological tests and cross-matching from hirudinised and native blood of the same donors gave identical results. Single clotting factors, but not global coagulation analytes, could be measured from hirudinised blood. Therefore, a universal hirudin-containing blood sampling tube could be designed for automated analysis of haematological, serological and clinical chemistry analytes.

Key words: Blood sampling tube; Blood anticoagulation; Hirudin.

Abbreviations: aPTT, activated prothrombin time; HBST, hirudinised blood sampling tube; ISE, ion-selective electrodes; PF4, platelet factor-4; TMA, thrombocyte microaggregates.

Introduction

Since ancient times, the leech has been known for its blood-letting capabilities. The responsible anticlotting properties of products secreted from salivary glands of the leech *Hirudo medicinalis* were first recognized in

1884 (1). Decades thereafter, crude anticoagulant compounds were isolated from leech (2) and systematically compared to other anticoagulants such as heparin (3). In the late 1950s, Markwardt purified leech anticoagulants and characterised their polypeptide structure (4). Recently, large-scale production of recombinant hirudin (5–10) has led to the development, preclinical evaluation and introduction into clinical trials of recombinant desulphated hirudin.

Natural and recombinant desulphated hirudin have similar biological and anticoagulant activities (11, 12). Natural hirudin is a single-chain, carbohydrate-free polypeptide composed of 65 amino acids (molecular weight: 7 kDa). The C-terminus of hirudin irreversibly binds to the fibrinogen recognition site of thrombin (13) and thus effectively blocks the transformation of fibrinogen to fibrin (14, 15). No cofactors are involved in this interaction. Hirudin also blocks the α -thrombin precursor meizothrombin (16).

Its highly specific interaction with a single factor of the complex clotting cascade makes hirudin an ideal anticoagulant for diagnostic blood tests. In addition, the use of hirudinised blood for automated blood count and, after subsequent centrifugation, hirudin plasma as a substrate to measure clinical chemistry analytes, would enable the design of a universal blood collecting tube. To test this hypothesis, we performed a feasibility study comparing the results of testing for diagnostic blood analytes from healthy individuals and patients using a single hirudinised blood sampling tube (HBST) and corresponding routine multiple blood sampling tubes.

Materials and Methods

Human subjects

Venous blood was taken from healthy blood donors (n=35) and hospitalised adults of all age groups (n=45). The patients suffered from different medical conditions including acute and chronic leukaemia, sepsis due to bacterial infections, renal and liver failure, diabetes mellitus, coronary heart disease, and myocardial infarction. The ratio of women to men was 3:5 and 1:1 for healthy volunteers and patients, respectively. All patients gave their informed consent to participate in this study.

Preparation of hirudinised blood sampling tubes (HBSTs)

Recombinant hirudin was expressed in the methylotrophic yeast, *Hansenula polymorpha* (10). Three purity grades of recombinant desulphato-hirudin were produced only differing in the content of N-terminally elongated or truncated forms. For grade I > 98%, for grade II > 70%, and for grade III > 70% of total protein represented hirudin molecules of the authen-

tic 65 amino acids in length, whereas the remaining protein fraction contained biologically active but slightly extended or truncated peptides. The majority of these protein impurities consisted of hirudin forms with an N-terminal extension (66 amino acids) or with C-terminal degradations (64, 63, 62, 56 amino acids). Contaminant yeast protein from the fermentation medium was neglectable. The different hirudin grades (Rhein Biotech GmbH, Duesseldorf, Germany) were used at comparable biological activity (anti-thrombotic units; ATU). Lyophilised hirudin (4.25 mg of each charge; specific anti-thrombotic activity (ATU) of 19,000, 18,800, and 18,300 per mg for the charges I-III, respectively) was dissolved in isotonic saline (0.9% NaCl) to prepare a hirudin working solution containing 100 ATU/ μ l (17). The working solution was kept refrigerated (4 °C). Aliquots of hirudin working solution were pipetted into plain 4 ml glass tubes thereby releasing the vacuum. For titration experiments, different volumes of hirudin working solution were pipetted into the glass tubes to prepare HBSTs with defined hirudin concentrations when filled with 4 ml venous blood. For all other experiments HBSTs containing 1000 ATU/ml hirudin were used. Plastic Vacutainer® blood sampling tubes (Becton Dickinson, Heidelberg, Germany) containing K₂EDTA- (4 ml tube; for blood cell count), fluoride as glycolysis inhibitor (3 ml tube; to assess serum glucose levels), clot activator and SST separation gel (8.5 ml serum tube; for measuring clinical chemistry and serologic analytes), or heparin (10 ml tube, for blood cell immunology), and a 7 ml glass tube with no additives (Becton Dickinson; for immunohaematological tests) were used for conventional blood sampling.

Blood sampling and sample preparation

Using a butterfly venipuncture system, blood was taken from a forearm vein. As a control, blood was drawn directly into a Vacutainer tube (Becton Dickinson) first. Thereafter, a sufficient volume of blood was drawn into a sterile 20 ml plastic syringe using the Luer-port of the already placed venipuncture system. Subsequently, 4 ml of blood (according to the syringes' scale) were transferred manually into each HBST. To speed up the procedure in order to prevent activation of the clotting cascade in the syringe, two skilled helpers were engaged in this process. Routine blood testing was performed within 6 hours after venipuncture. K₂-EDTA-tubes and HBSTs were moved gently to prevent thrombocyte microaggregation. Blood tubes were transported and stored at room temperature. Anticoagulated blood was taken out of the HBSTs to measure haematological analytes, first. To measure clinical chemistry analytes, HBSTs were centrifuged (Labofuge 400 E, 600 g; Heraeus, Osterode, Germany) to separate hirudin plasma.

Testing different hirudin concentrations for blood clotting

Blood samples from six healthy individuals were taken using HBST tubes with different hirudin concentrations. At defined time points after venipuncture (30 min, 4, 8, 12, 16, 20 and 24 hours), the tubes were gently shaken against light (transluminator) to visually check for complete blood clotting (++), macroscopically visible small agglutinations (+) or the absence of any signs of blood clotting (smooth blood flow on inner glass surface; 0). In addition, blood samples from healthy volunteers (n=3) were anticoagulated with hirudin of three different purity grades. From these samples, blood smears were prepared and stained at defined time points after sampling to analyse microscopically the presence of thrombocyte microaggregates (TMA). Sodium heparin- and K₂-EDTA-anticoagulated blood samples from the same donors served as controls.

Testing for haematological analytes

Automated blood count and differential was performed using a Coulter STK-R® cell counter (Beckman Coulter, Krefeld, Germany) according to manufacturer's instructions. In addition, regular blood smears were stained with May-Grünwald solution and analysed manually by light microscopy. Although the automated counters were optimized for K₂-EDTA-anticoagulated blood, both, K₂-EDTA-tubes and HBSTs were treated similarly.

Immunohaematological tests

A routine plate test was performed on centrifuged native or hirudinised blood. One drop (50 μ l) of anti-A, anti-B, anti-D, anti-CDE, Rh-control, anti-CcEe, and anti-Kell serum as well as test erythrocytes 0, A1, A2, and B (a drop of each) were placed into separate wells of a plastic tray. Antiserum was mixed with one drop of patient's blood, and test erythrocytes were mixed with two drops of patient's serum. After incubation at room temperature (5 min), agglutination was visually assessed and documented. In addition, immunohaematological tests were performed in glass tubes. First, patient's erythrocytes were re-suspended in 0.9% NaCl-solution. Two drops of patient's serum were placed into each of the numbered tubes 1–4, and one drop of patient's erythrocyte suspension was placed into each of the tubes 5–12. One drop of test erythrocytes group 0, A1, A2, and B was added to tubes 1–4, respectively, and one drop of anti-D, anti-CDE, Rhesus-control, anti-C, anti-c, anti-E, anti-e, and anti-Kell was added to tubes 5–12, respectively. The tubes were centrifuged for 20 s (Immunofuge, Biotest AG, Frankfurt/M, Germany). Agglutination was monitored visually. Reagents were from Biotest (A, B, 0), Immuncor (Rhesus antisera; Rödermark, Germany), Mast Diagnostika (Rhesus antisera; Rheinfeld, Germany), Dade-Behring (Liederbach, Germany; test erythrocytes), and Ortho Klinische Diagnostica (Neckargemünd, Germany; test erythrocytes).

Complete crossmatch including antibody detection test

Patient's blood was centrifuged (1000 g, 5 min). A suspension (approximately 4%) of donor (taken from a tube segment of a packed red cell unit) and recipient erythrocytes was prepared. The test was carried out as a routine Lis-Coombs (IC)-enzyme-test in glass tubes (incubated at 37 °C) and in centrifugation gel cards using the Dia-Med-ID Typing System (DiaMed Diagnostika, Cressier sur Morat, Switzerland) according to manufacturer's instructions. Reagents were from Dade-Behring AG (Liederbach, Germany) and Ortho Clinical Diagnostika GmbH (Neckargemünd, Germany) for the tube test and from Dia-Med for the gel cards. Agglutination in the tube test was monitored visually. After centrifugation (1000 g), gel cards were read using the ID-Reader (Dia-Med).

Testing for routine clinical chemistry analytes

After centrifuging HBSTs, 24 different clinical chemistry analytes were measured on hirudin plasma (analyser: Roche/Hitachi 917 System, Roche Diagnostics®, Mannheim, Germany). Test kits and reagents were from Roche Diagnostics®. All assays were performed at 25 °C (room temperature). The enzymatic UV-test (according to Warburg and Christian) was used to measure amylase (maltogenic method), α -hydroxybutyrate dehydrogenase (α HBDH; catalysis of α -ketobutyrate to lactate), alanine aminotransferase (ALT; catalysis of α -ketobutyrate and L-alanine to pyruvate followed by catalysis of pyruvate to lactate by LDH), aspartate aminotransferase (AST; catalysis of α -ketobutyrate and aspartate to pyruvate followed by catalysis of pyruvate to lactate by LDH), creatinine kinase

(CK; coupled UV-test), lactate dehydrogenase (LDH; catalysis of pyruvate to lactate), triglycerides (glycerate dehydrogenase test), and urea (urease/glutamate dehydrogenase test). Enzymatic colorimetric assays were used to measure alkaline phosphatase (AP; nitrophenol method), cholesterol (cholesterol oxidase method), γ -glutamyl transferase (γ GT; 5-amino-2-nitrobenzoate-test), glucose (glucose oxidase method), and uric acid (peroxidase reaction). Non-enzymatic photometry was used for measuring albumin (photometry with bromocresol-green dye), bilirubin (Jendrassik-Gróf method), calcium (chromogenic photometry), creatinine (Jaffe-reaction), free haemoglobin (cyanohaemoglobin method), phosphate (molybdenum-blue method), and total protein (Biuret reaction). Direct potentiometry using ion-selective electrodes (ISE) was employed to measure chloride, potassium (ISE with valinomycin membranes), and sodium. Pancreatic elastase was measured using an ELISA with monoclonal antibodies. The triolein turbidimetric method was used for lipase measurements. The coagulation tests thromboplastin time (Quick-test; coagulometric method), activated partial thromboplastin time (aPTT; coagulometric method), fibrinogen (immunologic method) and antithrombin III (ATIII; photometry using a chromogenic assay) were measured on venous blood from healthy individuals ($n=10$). No modifications were made to the settings of the analysers or to the test kits when tests on hirudin plasma were being carried out.

Statistical analyses

Standard deviation and paired t-tests were computed for all analytes. For repeated complete blood counts and clinical chemistry tests, mean standard deviation was calculated and expressed as percentage of the mean of repeated measurements. Correlation analysis according to Spearman (18) was performed in order to identify a correlation between measurements from hirudinised and routinely processed blood. In addition, simple linear regression analysis was employed. All statistical analyses were performed using the SPSS software (Version 6.1.1; SPSS Inc., USA).

Results

Hirudin concentration required to anticoagulate blood

Hirudin at a concentration of at least 300 ATU/ml was found effective in the prevention of blood clotting for at least 24 hours. As expected, heparin (75 IU/ml) and K_2 -EDTA (1.8 mg/ml) also prevented blood clotting (Table 1). Hirudin-anticoagulated blood (= 200 ATU/ml) could be readily analysed for cellular components in the automated blood counters (SKTS).

Formation of thrombocyte microaggregates (TMA)

Thrombocyte microaggregates, composed of approximately 3 to 10 aggregated thrombocytes, were found in the blood films of hirudinised blood. Therefore, TMA formation was systematically analysed using much higher hirudin concentrations and various hirudin purity grades. TMA formation was evaluated by an operator who estimated the frequency of TMA in 10 successive microscopic fields (630 \times) per single blood film. High hirudin concentrations corresponded to a substantially lower frequency of TMA. However, even very high hirudin concentrations (up to 2000 ATU/ml) could

not totally prevent TMA formation. At hirudin concentrations of 400 ATU/ml and above, TMA formation was rare and the average size of a single TMA was relatively small. In films of K_2 -EDTA-anticoagulated blood, TMA formation was much less frequent compared to hirudinised blood (400 ATU/ml). In contrast, films of heparin-anticoagulated blood disclosed much more TMA formation compared to hirudinised blood (400 ATU/ml; Table 2).

The anticoagulant activity of three hirudin charges (grades I, II, III) differing in purity, was similar (data not shown), and no differences in the rate of TMA formation was found between hirudin of high, median and low purity grade (Table 2).

Reproducibility of the automated complete blood counts in hirudinised and K_2 -EDTA-anticoagulated blood

To analyse whether hirudinised blood (1000 ATU/ml) was sufficiently anticoagulated for automated blood cell counting, five successively repeated blood counts were performed from hirudinised and K_2 -EDTA-anticoagulated blood from the same donors ($n=19$). The measurements were performed 6 hours after venipuncture. The standard deviation was significantly larger for the measurements of erythrocytes, haemoglobin and eosinophils when hirudinised, instead of K_2 -EDTA-anticoagulated blood was used. However, the absolute variation of repeated blood cell counting was very low (data not shown). For cells normally present in low numbers (monocytes, eosinophils, and basophils), the average standard deviation in percent of the mean of repeated automated cell counts was higher in hirudinised as compared to K_2 -EDTA-anticoagulated blood (Table 3).

Comparative analysis of complete blood counts obtained from K_2 -EDTA- and hirudin-anticoagulated blood

Blood from healthy volunteers ($n=35$) and hospitalised patients ($n=45$) was drawn into K_2 -EDTA-tubes and HB-STs through the same venipuncture set. The tubes were processed routinely within 6 hours after venipuncture. Automated complete blood counts and manual differentials from both sampling tubes were compared.

Significant differences were found between the means of the measurements of leukocytes and thrombocytes obtained from K_2 -EDTA- and hirudin-anticoagulated blood (t-test), whereas the measurements of all other haematological variables were similar. However, these differences were small in absolute terms and thus not clinically relevant. Furthermore, a significant correlation (Spearman's test) was found between the automated measurements from hirudinised and K_2 -EDTA-anticoagulated blood for all blood cells and haemoglobin. The significance level of the correlation was substantially lower for monocytes ($p=0.5$) and basophils ($p=0.5$) compared to the other cellular blood constituents (Table 3). Occasionally, some monocytes were mistaken for basophils during automated blood

Tab. 1 Anticoagulant effect of recombinant desulphated hirudin, heparin and K₂-EDTA on venous blood of healthy volunteers measured at different time points after venipuncture.

Time points after sampling	Volunteers (n=6)	Recombinant desulphated hirudin (high-grade purity) in ATU/ml							Heparin (75 IU/ml)	K ₂ -EDTA (1 mg/ml)
		0	50	100	150	200	250	300		
30 minutes	SP	++	+	0	0	0	0	0	0	0
	JR	++	+	0	0	0	0	0	0	0
	HM	++	+	0	0	0	0	0	0	0
	NB	++	+	+	nd	0	nd	0	0	0
	AS	++	+	0	nd	0	nd	0	0	0
	SV	++	+	+	nd	0	nd	0	0	0
4 hours	SP	++	++	+	0	0	0	0	0	0
	JR	++	++	+	0	0	0	0	0	0
	HM	++	++	+	0	0	0	0	0	0
	NB	++	+	+	nd	0	nd	0	0	0
	AS	++	+	+	nd	+	nd	0	0	0
	SV	++	+	+	nd	0	nd	0	0	0
8 hours	SP	++	++	+	0	0	0	0	0	0
	JR	++	++	+	+	0	0	0	0	0
	HM	++	++	+	0	0	0	0	0	0
	NB	++	nd	+	nd	0	nd	0	+	0
	AS	++	nd	+	nd	+	nd	0	0	0
	SV	++	nd	+	nd	0	nd	0	0	0
12 hours	SP	++	++	++	0	0	0	0	0	0
	JR	++	++	++	+	0	0	0	0	0
	HM	++	++	++	0	0	0	0	0	0
	NB	++	+	+	nd	0	nd	0	+	0
	AS	++	+	+	nd	+	nd	0	0	0
	SV	++	+	+	nd	+	nd	0	0	0
16 hours	SP	++	++	++	+	0	0	0	0	0
	JR	++	++	++	+	0	0	0	0	0
	HM	++	++	++	+	0	0	0	0	0
	NB	++	++	+	nd	+	nd	0	+	0
	AS	++	++	+	nd	+	nd	+	0	0
	SV	++	+	+	nd	+	nd	0	0	0
20 hours	SP	++	++	++	+	0	0	0	0	0
	JR	++	++	++	+	0	0	0	0	0
	HM	++	++	++	+	0	0	0	0	0
	NB	++	nd	nd	nd	nd	nd	nd	+	0
	AS	++	nd	nd	nd	nd	nd	nd	0	0
	SV	++	nd	nd	nd	nd	nd	nd	0	0
24 hours	SP	++	++	++	+	+	0	0	0	0
	JR	++	++	++	++	+	+	0	0	0
	HM	++	++	++	+	0	0	0	0	0
	NB	++	++	+	nd	+	nd	0	+	0
	AS	++	++	+	nd	+	nd	+	0	0
	SV	++	+	+	nd	+	nd	+	0	0

The venous blood (4 ml) from six healthy volunteers (initials: SP, JR, HM, NB, AS, SV) was drawn into HBSTs containing varying amounts of desulphated hirudin. In controls, blood was drawn into manufactured K₂-EDTA- and heparin-contain-

ing blood sampling tubes. All tubes were visually monitored for clotting and small agglutinations. ++: complete blood clotting; +: small agglutinations floating in non-clotted blood; 0: no visible blood clotting, no small agglutinations; nd: not done.

cell counting, resulting in incorrectly high basophil counts. This phenomenon occurred more frequently when hirudinised blood was measured compared to

K₂-EDTA-anticoagulated blood (in 7 and 2 out of 80 samples, respectively), and was usually resolved by repeated measurements. Linear regression analyses

Tab. 2 Formation of thrombocyte microaggregates (TMA) in hirudinised blood at room temperature at defined time points after sampling.

Time points after sampling	Hirudin purity grade	Volunteers (n=3)	Recombinant desulphated hirudin in ATU/ml venous blood						Heparin (75 IU/ml)	K ₂ -EDTA (1 mg/ml)	
			200	300	400	600	800	1000			2000
6 hours	High	V1	**	**	*	*	0	*	0		
		V2	**	**	*	0	0	0	0	**	0
		V3	**	**	*	*	*	0	*		
	Medium	V1	**	**	**	0	0	0	0		
		V2	**	**	*	*	0	0	0	**	0
		V3	**	**	*	*	0	0	*		
	Low	V1	**	**	*	0	*	0	0		
		V2	**	*	*	*	*	*	0	**	0
		V3	**	**	*	*	0	0	0		
12 hours	High	V1	**	*	*	*	0	0	0		
		V2	**	**	*	0	0	*	0	**	0
		V3	*	**	*	*	*	0	0		
	Medium	V1	**	**	*	*	*	0	0		
		V2	**	**	*	*	0	*	*	**	0
		V3	**	**	*	0	0	0	0		
	Low	V1	**	**	*	*	*	0	0		
		V2	**	**	*	*	*	0	*	**	0
		V3	**	**	**	*	*	*	0		
18 hours	High	V1	**	**	*	*	*	0	0		
		V2	**	**	*	*	*	*	*	**	0
		V3	**	**	*	*	*	*	0		
	Medium	V1	**	**	*	*	*	0	*		
		V2	**	**	**	*	*	*	*	**	0
		V3	**	**	*	*	*	*	*		
	Low	V1	**	**	*	*	*	*	*		
		V2	**	**	*	*	*	0	0	**	0
		V3	**	**	*	0	*	*	*		
24 hours	High	V1	**	**	*	*	*	*	*		
		V2	**	*	*	*	*	*	*	**	0
		V3	**	**	*	*	*	*	*		
	Medium	V1	**	**	*	*	*	*	*		
		V2	**	**	**	*	*	*	*	**	0
		V3	**	**	*	*	*	*	*		
	Low	V1	**	**	*	*	*	*	*		
		V2	**	**	*	*	*	*	*	**	*
		V3	**	**	*	*	*	*	*		

The blood from healthy volunteers was hirudinised using different purity grades and varying concentrations of recombinant desulphated hirudin. Prepared HBSTs were filled with 4 ml of venous blood. Manufactured heparin- and K₂-EDTA-containing blood sampling tubes served as controls. At defined time points

after sampling, a blood smear was prepared from each blood sampling tube and screened microscopically for the presence of TMA. **: at least one TMA in every other microscopic field (power 630 ×); *: at least one TMA in 1–4 of 10 microscopic fields; 0: no TMA found in 10 of 10 successive microscopic fields.

showed, that the complete blood counts on EDTA-anticoagulated blood were predictable by the respective

measurements obtained from hirudinised blood (significance level $p < 0.001$; Table 3).

Tab. 3 The automated blood counts from K₂-EDTA-anticoagulated (E) and hirudinised blood (H) from the same donors (n=77–80).

Analyte	Anti-coagulant	Percent standard deviation of test (%) [#]	Mean of absolute results	Mean difference between substrates (%)	Standard deviation	2-Sided t-test	Correlation coefficient (Spearman)	Linear regression β -coefficient
Erythrocytes/pl	E	0.5	4.01	-0.02	0.86	0.677	0.990***	0.995***
	H	0.9	4.01	(0.02)	0.93			
Leukocytes/nl	E	1.2	9.46	0.70	10.25	0.000***	0.946***	0.994***
	H	4.4	8.77	(7.4)	9.36			
Thrombocytes/nl	E	2.4	210.33	8.98	72.60	0.001***	0.909***	0.958***
	H	8.8	201.35	(4.26)	70.21			
Haemoglobin g/dl	E	0.5	12.35	-0.01	2.55	0.679	0.989***	1.000***
	H	0.8	12.35	(0.08)	2.58			
Neutrophils (%)	E	1.3	65.07	1.11	17.48	0.531	0.874***	0.765***
	H	9.3	63.97	(1.71)	20.55			
Lymphocytes (%)	E	2.1	24.09	0.25	15.98	0.096	0.965***	0.917***
	H	4.5	24.34	(1.04)	14.55			
Monocytes (%)	E	9.1	7.74	-0.44	5.30	0.790	0.510*	0.936***
	H	27.4	8.18	(-5.62)	11.20			
Eosinophils (%)	E	12.6	2.45	-0.02	3.54	0.905	0.878***	0.976***
	H	13.7	2.45	(-0.86)	3.40			
Basophils (%)	E	60.0	0.63	-0.28	0.75	0.373	0.241*	0.100***
	H	90.7	0.91	(-44)	1.33			

[#] Average standard deviation in percent of mean of repeated automated blood counts (five successive repeats) from

healthy volunteers (n=19); significant on level *** p<0.001, ** p<0.01, and * p<0.5.

Blood smears from the HBSTs and K₂-EDTA-tubes (n=69) that were used to obtain automated blood counts were analysed microscopically. The mean of basophil measurements was significantly higher (t-test) when automated cell counts were compared with manual differentials, irrespective of the anticoagulant used. Interestingly, the mean basophil count was not statistically different when manual EDTA differentials were compared to manual hirudin differentials. In manual EDTA differentials significantly more neutrophils and less lymphocytes were counted compared to manual hirudin differentials. Similar differences were observed when manual and automated EDTA differentials were compared. Finally, manual differentials revealed a significantly higher mean lymphocyte count when compared to automated hirudin differentials. These differences were low in terms of absolute cell numbers and thus clinically irrelevant. No significant differences were found, except for basophils, when manual EDTA differentials (gold standard) were compared to automated hirudin differentials. Furthermore, significant correlations (Spearman's test) were found between the measurements of neutrophils, lymphocytes, monocytes, and eosinophils on hirudinised and K₂-EDTA-anticoagulated blood. Only the basophil count did not correlate, both when automated and manual differential from K₂-EDTA-anticoagulated blood were assessed (Table 4).

Immunohaematological tests and screening for irregular antibodies

Native clotted and hirudinised blood (1000 ATU/ml) from the same patients (n=10) was taken for immuno-

haematological tests using a standard tube test. The results of blood group typing and complete cross-matching including antibody detection on hirudinised blood were indistinguishable from those on native blood (standard procedure). Using the DiaMed-IP device, automated microtyping of blood groups, cross-matching and antibody detection on hirudinised blood samples (n=5) was possible, and the results were identical to those obtained from native blood of the same blood donors (data not shown).

Repeated measurements of clinical chemistry analytes on hirudin plasma and serum

Blood from healthy volunteers (n=3) was drawn into HBSTs (1000 ATU/ml) and serum tubes. The tubes were routinely processed within 6 hours. To detect differences in the reproducibility of the measurements of 24 clinical chemistry analytes, serum and hirudin plasma from the same patients were analysed five times successively. The standard deviation of repeated measurements from serum and hirudin plasma was not significantly different for all 24 clinical chemistry analytes (data not shown), and the averaged standard deviation expressed as percentage of the mean of repeated measurements was similar for serum and hirudinised blood (Table 5).

Clinical chemistry analytes measured in hirudin plasma and serum

Hirudin plasma and serum from healthy volunteers (n=25) and from hospitalised patients (n=34–49) was used for measuring 24 clinical chemistry analytes. For

Tab. 4 Blood differential counts performed on K₂-EDTA-anticoagulated and hirudinised blood from the same donors (n=69).

Analyte	Anticoagulation/ procedure	Mean of absolute values	Mean difference between sub- strates (%)	Standard deviation	2-Sided t-test	Correlation coefficient (Spearman)
Manual EDTA- vs. manual hirudin differentials						
Neutrophils (%)	E/m	66.46	4.49	15.74	0.000***	0.900***
	H/m	61.97	(6.75)	17.73		
Lymphocytes (%)	E/m	24.48	-4.9	13.78	0.000***	0.895***
	H/m	29.38	(-20.02)	16.45		
Monocytes (%)	E/m	6.71	0.61	4.50	0.085	0.739***
	H/m	6.10	(9.10)	4.85		
Eosinophils (%)	E/m	1.81	-0.38	2.03	0.138	0.633***
	H/m	2.19	(-21)	2.37		
Basophils (%)	E/m	0.19	0.09	0.70	0.380	0.106
	H/m	0.10	(47)	0.52		
Manual EDTA- vs. automated EDTA differentials						
Neutrophils (%)	E/m	66.46	1.39	15.74	0.044*	0.963***
	E/a	65.07	(2.1)	17.63		
Lymphocytes (%)	E/m	24.48	0.4	13.78	0.997	0.924***
	E/a	24.08	(1.6)	16.03		
Monocytes (%)	E/m	6.71	-1.03	4.50	0.169	0.669***
	E/a	7.74	(-15.35)	5.48		
Eosinophils (%)	E/m	1.81	-1.64	2.03	0.092	0.725***
	E/a	2.45	(-90.61)	3.67		
Basophils (%)	E/m	0.19	-0.44	0.77	0.000***	0.073
	E/a	0.63	(-231.6)	0.69		
Manual hirudin- vs. automated hirudin differentials						
Neutrophils (%)	H/m	61.97	2	17.73	0.017**	0.863***
	H/a	63.97	(3.26)	19.34		
Lymphocytes (%)	H/m	29.38	5.04	16.45	0.000***	0.880***
	H/a	24.34	(17.15)	14.60		
Monocytes (%)	H/m	6.10	-2.08	4.85	0.208	0.573***
	H/a	8.18	(-34.0)	11.55		
Eosinophils (%)	H/m	2.19	-0.29	2.37	0.379	0.6292***
	H/a	2.48	(-13.24)	3.51		
Basophils (%)	H/m	0.10	-0.81	0.52	0.000***	0.104
	H/a	0.91	(-810.0)	1.34		
Manual EDTA- vs. automated hirudin differentials						
Neutrophils (%)	E/m	66.46	2.49	19.34	0.537	0.851***
	H/a	63.97	(3.75)	15.74		
Lymphocytes (%)	E/m	24.48	0.14	14.60	0.052	0.915***
	H/a	24.34	(0.57)	13.78		
Monocytes (%)	E/m	6.71	-1.47	11.55	0.418	0.563***
	H/a	8.18	(-21.91)	4.50		
Eosinophils (%)	E/m	1.81	-0.67	3.51	0.068	0.624***
	H/a	2.48	(-37.02)	2.03		
Basophils (%)	E/m	0.19	-0.72	1.34	0.001**	0.293*
	H/a	0.91	(-378.95)	0.69		

E: EDTA-blood, H: hirudin-blood, m: manual measurement, a: automated measurement; significant on level *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

some analytes, a significant difference in the mean of the measurements (t-test) was found when results from serum were compared to those obtained from

hirudinised plasma. However, with the exception of potassium, phosphate, lipase, and bilirubin, the absolute differences in the mean values were low (less

Tab. 5 The measurements of clinical chemistry analytes on serum (S) and hirudin plasma (H) from the same blood donors (n=64–75).

Analyte (unit)	Anticoagulant	Percent standard deviation of test (%)#	Mean of absolute values	Mean of difference serum/hirudin (%)	Standard deviation	2-Sided t-test	Correlation coefficient (Spearman)	Linear regression β -coefficient
α -Amylase (U/l)	S	3.6	105.75	1.61	171.38	0.022*	0.996***	1.000***
	H	0.7	103.92	(1.52)	165.99			
α -HBDH (U/l)	S	0.7	148.38	1.05	125.91	0.202	0.983***	0.998***
	H	0.0	147.18	(0.70)	123.07			
Albumin (g/l)	S	1.3	38.43	0.11	7.54	0.271	0.986***	0.993***
	H	0.7	38.31	(0.23)	7.32			
ALT (U/l)	S	8.3	23.33	0.4	38.58	0.158	0.997***	0.999***
	H	4.3	22.94	(1.71)	36.95			
AP (U/l)	S	1.7	139.51	-1.51	150.03	0.334	0.988***	0.996***
	H	0.7	142.45	(-1.08)	143.50			
AST (U/l)	S	11	18.03	-0.5	3.62	0.086	0.990***	0.999***
	H	5.3	17.72	(-2.77)	3.49			
Bilirubin (μ mol/l)	S	18.3	17.31	-0.95	36.79	0.045*	0.918***	0.998***
	H	4.7	18.25	(-5.48)	40.12			
Calcium (mmol/l)	S	0.3	2.36	-0.02	0.29	0.51	0.825***	0.929***
	H	0	2.35	(-0.12)	0.29			
Chloride (mmol/l)	S	0.3	105.78	0.82	0.64	0.003	0.878***	0.881***
	H	0.0	104.85	(0.78)	0.60			
Cholesterol (mmol/l)	S	1.3	4.76	0.11	0.19	0.000***	0.994***	0.994***
	H	0.3	4.64	(2.31)	0.18			
Creatine kinase (U/l)	S	1.3	60.22	2.47	112.04	0.000***	0.997***	1.000***
	H	0.3	57.40	(4.10)	107.34			
Creatinine (μ mol/l)	S	2	146.55	-3.77	121.93	0.961	0.977***	0.998***
	H	0.3	148.58	(-2.57)	120.98			
γ -GT (U/l)	S	5	73.09	2.15	146.89	0.007**	0.973***	0.998***
	H	2.3	69.49	(2.94)	146.25			
Glucose (mmol/l)	S	1.3	6.65	0.22	0.41	0.000***	0.922***	0.992***
	H	0.3	6.30	(3.30)	0.40			
Lactate dehydrogenase (U/l)	S	0.3	239.31	3.03	214.68	0.162	0.975***	0.998***
	H	0.0	235.85	(1.27)	201.65			
Lipase (U/l)	S	11	154.75	8.81	293.43	0.000***	0.980***	0.999***
	H	11.3	144.72	(5.70)	290.68			
Pancreatic amylase (U/l)	S	5.7	61.28	0.78	139.94	0.028*	0.995***	1.000***
	H	3	60.38	(1.27)	137.11			
Phosphate (mmol/l)	S	0.3	1.188	0.07	0.04	0.000***	0.963***	0.967***
	H	0.3	1.095	(5.89)	0.04			
Potassium (mmol/l)	S	0.3	4.14	0.39	0.63	0.000***	0.890***	0.942***
	H	0.0	3.77	(9.42)	0.61			
Sodium/ (mmol/l)	S	0.0	138.75	1.18	4.56	0.000***	0.886***	0.815***
	H	0.0	137.56	(0.85)	3.84			
Total protein (g/l)	S	0.3	70.59	-2.84	11.53	0.000***	0.911***	0.968***
	H	0.0	73.47	(-4.02)	11.88			
Triglyceride (mmol/l)	S	1	1.88	0.06	1.40	0.544	0.941***	0.743***
	H	1	1.97	(3.19)	1.70			
Urea (mmol/l)	S	2.3	22.26	0.10	66.60	0.084	0.996***	0.999***
	H	2.7	21.06	(0.45)	62.64			
Uric acid (μ mol/l)	S	0.0	355.86	5.04	172.18	0.000***	0.996***	0.999***
	H	0.3	350.12	(1.42)	169.12			

Average standard deviation in percent of mean of repeated automated complete blood counts (three successive repeats) from healthy volunteers (n=3); significant on level *** p<0.001, **

p<0.01, and * p<0.5. HBDH: α -hydroxybutyrate dehydrogenase, ALT: alanine aminotransferase, AP: alkaline phosphatase, AST: aspartate aminotransferase, γ -GT: γ -glutamyl transferase

than 5%) and therefore clinically not relevant. The statistically significant differences in the measurements of other analytes can probably be explained by minimal

anticoagulant effects on an instrument of very good precision. Potassium, phosphate and lipase were consistently lower, whereas bilirubin and, to a lesser ex-

tent, total protein were higher in hirudin plasma compared to serum. However, a strong correlation was found between the measurements of serum and hirudin plasma for all 24 analytes (Table 5). Also, linear regression analysis demonstrated that the measurements of serum clinical chemistry analytes were predictable by the respective measurements obtained from hirudin plasma ($p < 0.001$; Table 5).

In healthy volunteers ($n=8$), free haemoglobin was much lower in hirudin plasma compared to serum. Although all measurements were within normal range, the mean free haemoglobin concentration in the volunteers' serum was 96.76 mg/dl (range 38.3 to 133 mg/dl) compared to 28.61 mg/dl (range 2.18 to 65.18 mg/dl) when hirudin plasma was analysed instead.

In the comprehensive coagulation tests, activated prothrombin time (aPTT) and thromboplastin time, clotting of hirudinised blood ($n=10$) could not be achieved. However, single clotting factors, fibrinogen and ATIII, could be analysed from hirudinised blood and gave sound results (data not shown).

Discussion

Recombinant desulphated hirudin, a direct antithrombin, is presently under extensive clinical investigation for a variety of medical indications such as preventing venous thromboembolism (19–21), acute coronary artery syndromes (22–28), and lipopolysaccharide-induced shock (29). Further, desulphated hirudin is sometimes given to patients who are treated with continuous haemodialysis (30, 31) and when severe heparin-induced thrombocytopenia (32–35) is diagnosed. However, very little published data is available on the use of hirudinised blood for diagnostic purposes. We performed a feasibility study to compare the results of automated complete blood count and clinical chemistry analyses on hirudinised blood with those obtained on routinely processed blood. The major objective of this study was to find out whether hirudin would interfere with automated blood counting or automated clinical chemistry analysis.

The desulphated hirudin concentration of at least 300 ATU/ml was found to adequately prevent blood clotting for at least 24 hours. Contrary to our observations, Stocker reported that 1000 ATU desulphated hirudin per ml were necessary to anticoagulate blood for at least 24 hours (36). Although automated blood count could be performed using hirudinised blood at 300 ATU/ml, we frequently found TMA formation in the manual differentials, indicating activation of thrombocytes. In order to optimise blood hirudin concentration for diagnostic purposes, we systematically studied TMA formation at different time points after venipuncture, and at differing hirudin concentrations and purity grades. TMA formation was not associated with elapsed time after venipuncture, and it was independent of the particular purity grades of desulphated hirudin. Although some TMA formation was still detectable when blood was hirudinised using high

hirudin concentrations (1000 and 2000 ATU/ml), we believe that at a concentration of at least 400 ATU hirudin per ml is suitable for diagnostic purposes. Heparin-anticoagulated blood showed a much stronger TMA formation compared to hirudinised blood, whereas in K_2 -EDTA-anticoagulated blood TMA formation took place rarely. These observations are in line with the results of others who found that anticoagulants stimulate thrombocytes to release platelet factor-4 (PF4). Heparin caused an approximately 5-fold, and hirudin and citrate a 2-fold increase in the release of PF4 compared to EDTA. Based on these findings, it seems that heparinised and, to a lesser extent, hirudinised blood has a propensity for spontaneous thrombocyte activation (37). However, TMA formation could simply be caused by the prolonged contact of blood with thrombogenic surfaces such as the sterile plastic syringe that we used to distribute blood into hirudin-containing sampling tubes. To resolve this issue, further experiments with manufactured hirudin-containing blood sampling tubes should be performed. Also, immunological phenomena are sometimes involved in thrombocyte activation. Studies on patients with anticoagulant-induced *in vitro* pseudothrombocytopenia suggest that anticoagulant-facilitated autoantibody binding to the GpIIb/IIIa complexes mimics binding of physiologic ligands such as fibrinogen or fibronectin, thus causing platelet activation and aggregation. In such patients, K_2 -EDTA prominently reduces platelet count by inducing TMA (platelet clotting), followed by sodium oxalate and heparin, whereas hirudin has a moderate effect only (38).

In further experiments, blood was anticoagulated with 1000 ATU hirudin per ml to facilitate detection of possible interactions between hirudin and automated blood counting as well as automated analysis of clinical chemistry analytes. Although automated complete blood counts could be performed reproducibly from hirudinised blood, repeated measurements of erythrocytes, haemoglobin and eosinophils showed a significantly greater variation when hirudinised blood was used instead of K_2 -EDTA-anticoagulated blood. However, the absolute differences in the results were very low and thus clinically irrelevant. For all blood cells, a significant correlation was found between the measurements on K_2 -EDTA-anticoagulated and hirudinised blood, even when blood of severely ill patients (*e.g.* with leukaemia, sepsis or thrombocytopenia) was tested. Regression analysis demonstrated predictability of the K_2 -EDTA complete blood count from the respective measurements on hirudinised blood. In addition, the measurements of automated and microscopic manual differentials on hirudinised and on K_2 -EDTA-anticoagulated blood showed a significant correlation for all cells but basophils. Since basophils normally account for less than 2% of the nucleated blood cells, a small difference in the count has a strong statistical impact.

Occasionally, a misclassification between basophils and monocytes occurred in automated differentials. Although this is also known for K_2 -EDTA-anticoagulated

blood, this phenomenon occurred more frequently with hirudinised blood. This discrimination failure between basophils and monocytes, which possibly also explains why automated differentials always have a higher basophil count than normal ones, irrespective of the anticoagulant, can be resolved by performing three successive measurements. If both repeats indicate plausible values for basophils and monocytes, the initial measurement is to be deleted and the first repeat taken as the correct count (most cases). In all other cases, a new blood sample has to be analysed. Other direct thrombin inhibitors such as PPACK (D-phenylalanyl-L-propyl-arginine chloromethylketone) and argatroban were used for automated blood cell counting. However, in approximately half of the samples, neutrophils, lymphocytes, monocytes and basophils were not recognised in PPACK- or argatroban-anticoagulated blood (Coulter Counter STK-R; 39). We never encountered this problem using hirudin as an anticoagulant.

Immunohaematological tests were possible using hirudinised blood. We did not find any indication that hirudin interferes with manual or automated immunohaematological tests or cross-matching.

Clinical chemistry analytes could be reproducibly measured from hirudin plasma and serum. The standard deviations of repeated automated measurements of these analytes from hirudin plasma and serum were indistinguishable. The average difference in the mean results for potassium, phosphate, lipase and bilirubin exceeded 5% when results from serum and hirudinised plasma were compared. Potassium, phosphate and lipase were consistently higher in serum compared to hirudin plasma. This known phenomenon (40) is probably attributable to volume differences between plasma and serum (approximately 4% in favour of plasma) and to platelet activation and blood cell lysis during coagulation (41). This explanation is supported by our observation that hirudin plasma contains less free haemoglobin than serum. Further, it should be kept in mind that thrombocytosis can cause falsely elevated potassium concentration (42). Bilirubin was consistently lower in serum compared to hirudinised plasma. This observation could possibly be attributed to binding phenomena between bilirubin and proteins of the clotting cascade. Total protein was found consistently higher in hirudinised plasma compared to serum due to the depletion of serum from clotting proteins. However, for all clinical chemistry analytes, a highly significant correlation was observed between the measurements on serum and hirudin plasma of the same blood donors, and linear regression analysis revealed that the results of measurements of serum clinical chemistry analytes could be predicted by the respective measurements on hirudin plasma. Hirudin plasma could not be used for comprehensive coagulation tests (prothrombin test; Quick-test and aPTT), but single clotting factors fibrinogen and ATIII were measurable.

Taken together, these findings enable the design of a single universal blood sampling tube containing hirudin as the only anticoagulant, to be used for automated blood counting and, after subsequent centrifu-

gation, for automated clinical chemistry and serologic blood group testing. The use of such a unique blood sampling tube could reduce iatrogenic anaemia (43, 44) and initiate a redesign of instruments and laboratory workflow to increase cost-effectiveness (45, 46).

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