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DETERMINATION OF AMSACRINE IN HUMAN NUCLEATED HEMATOPOIETIC CELLS

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SUMMARY

A new method has been developed for the determination of amsacrine (AMSA) in human nucleated hematopoietic cells. In order to prevent efflux during the cell separation procedure, white blood cells (WBCs) were separated from red blood cells by dextran sedimentation, leaving the WBCs in their natural environment. After cell counting, pelletting the cell suspension and correcting for the admixture of supernatant, AMSA was extracted from the WBCs and determined by high-performance liquid chromatography. Linearity of extraction was observed up to $40 \cdot 10^6$ cells. The inter-assay variation was 4.7%. Plasma and cellular concentrations were measured in five patients at the end of a 3-h infusion of 100 mg/m^2 AMSA. A pharmacokinetic study of plasma and cellular AMSA concentrations up to 19 h after infusion was carried out. AMSA concentrations in WBCs correlated well with the plasma levels ($n=20$, $r=0.967$) with an accumulation factor compared to the plasma concentration of 2.6–9.8 in the patients studied. The method described is useful for studying cellular pharmacokinetics of AMSA in man.

INTRODUCTION

Amsacrine, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (AMSA), is a recently developed cytostatic agent, which is active against acute leukemia [1,2]. The plasma pharmacokinetics have been studied in patients treated for leukemia using [^{14}C] AMSA [3] and high-performance liquid chromatography (HPLC) [4]. However, the pharmacokinetics of AMSA in human hematopoietic cells have not been studied, neither in vivo nor in vitro, although such data are more relevant. Cellular pharmacokinetics of radiolabeled AMSA in cultured cells showed a rapid uptake and efflux of the drug [5–7]. To determine AMSA concentrations

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in white blood cells (WBCs), these have to be separated from the red blood cells (RBCs) and from plasma. Because commonly used cell separation methods such as density-cut, gradient or counter-flow centrifugation and specific lysis of RBCs are accompanied by a rapid efflux of AMSA out of the hematopoietic cells, a new method was developed to determine cellular AMSA concentrations after in vivo drug administration. Dextran sedimentation of RBCs [8], leaving the WBCs in their natural environment and avoiding dilutions and wash steps, provided good results.

EXPERIMENTAL

Drugs and chemicals

AMSA in N,N-dimethylacetamide, the lactic acid solution, and the AMSA analogue N-5-dimethyl-9-[(2-methoxy-4-methyl-sulfonylamino)phenylamino]-4-acridinecarboxamide (CI-921) used as internal standard were kindly supplied by Warner-Lambert/Parke-Davis (Ann Arbor, MI, U.S.A.). Methanol, acetonitrile (both from LAB-SCAN, Dublin, Ireland) were UV grade. Triethylamine, sodium tetraborate (both from Sigma, St. Louis, MO, U.S.A.), phosphoric acid, anhydrous diethyl ether, *n*-hexane and hydrochloric acid (all from Merck, Darmstadt, F.R.G.) were of analytical grade. Dextran (MW 250 000) was obtained from the N.P.B.I. (Emmer-Compascuum, The Netherlands).

Standard solutions

AMSA (50 mg/ml) was diluted with lactic acid (0.035 *M*) to a concentration of 5 mg/ml, diluted with a 5% glucose solution to a stock solution of 50 μg/ml and stored at −20°C. A stock solution of the internal standard (CI-921), 250 μg/ml, was prepared in methanol, diluted with a 5% glucose solution to 5 μg/ml and stored at −20°C. Dextran was dissolved in distilled water to a concentration of 25% (w/v) and stored at 4°C. A 1.0 *M* stock solution of triethylamine phosphate (TEAP) was made by adding 13.9 ml triethylamine to 60 ml distilled water, adjusting to pH 3.0 with phosphoric acid (85%) and diluting to 100 ml with distilled water.

Preparation of cell suspensions from peripheral blood

For validation studies, purified WBC suspensions were prepared. Blood was layered over a Ficoll-Isopaque (sp.gr. 1.085) gradient and centrifuged for 20 min at 850 *g* and 18°C. The interphase, containing purified WBCs, was taken off, washed twice with a phosphate-buffered saline (PBS) solution and finally resuspended in AB0 compatible plasma.

Blood samples for AMSA determination were taken in heparinized glass tubes. The mean cell volume (MCV) of the RBCs, hematocrit (Ht) and cell numbers were determined with an H1 analyser (Technicon, Tarrytown, NY, U.S.A.). From each blood sample 500 μl were taken for the determination of the AMSA concentration in whole blood ($AMSA_{Bl}$). A 1.5-ml aliquot was centrifuged at 850 *g* for 10 min, and 500 μl of plasma were used for the determination of AMSA concentration in plasma ($AMSA_{Pl}$). Of the remainder, 24 parts of whole blood were

mixed with 1 part of dextran solution in order to obtain a selective sedimentation of the RBCs. After sedimentation during 30 min at room temperature, the top layer, consisting of a WBC-rich cell suspension in plasma, was transferred to a polypropylene tube. WBCs and RBCs were counted with a Coulter counter (Coulter Electronics ZF 8108, Harpenden, U.K.).

A known amount of cell suspension was pipetted into preweighed tubes. After centrifugation, the supernatant was removed, and a sample of 500 μl was stored for the determination of the AMSA concentration in the supernatant ($AMSA_{Sup}$). The number of cells in the $AMSA_{Sup}$ was negligible.

The tube with the cell pellet was weighed again to determine the remaining volume of supernatant in the pellet. PBS (500 μl) was added and the tube was vortexed after adding a small glass bead. This sample was stored for the determination of AMSA in the cell pellet ($AMSA_{Cell}$). All samples were stored in two separate tubes at $-20°C$ until extraction.

Extraction

Aliquots of 500 μl representing either $AMSA_{Bl}$, $AMSA_{Pl}$, $AMSA_{Sup}$, or $AMSA_{Cell}$ were extracted, and the AMSA concentrations were determined with the HPLC method described by Jurlina and Paxton [9] with slight modifications. After addition of 100 μl of internal standard to control extraction and adjustment to pH 3.0–4.0 by hydrochloric acid (0.5 *M*), endogenous plasma or cellular compounds were removed by hexane extraction. The samples were adjusted to pH 9.0 with saturated sodium tetraborate, and AMSA and the internal standard were extracted with anhydrous diethyl ether. The organic layer was taken off and dried under air at 37°C. The residue was dissolved in 200 μl methanol–148 m*M* phosphoric acid (1:1, v/v) and stored at $-20°C$ until analysis by HPLC. Prior to HPLC, the samples were centrifuged at 6000 *g* for 10 min to remove precipitates.

High-performance liquid chromatography

The chromatographic system consisted of an isocratic HPLC pump (SP8770, Spectra-physics, Berkeley, CA, U.S.A.), an injection valve (AH 60, Valco, Houston, TX, U.S.A.) with a 100-μl injection loop, a LiChrosorb RP-8 guard column (50×4.6 mm I.D.) and a reversed-phase analytical column (150×4.6 mm I.D.) packed with silica gel Cp-Spher C_8, particle size 8 μm (Chrompack, Middelburg, The Netherlands). The wavelength of the UV detector (Pye Unicam, LC-UV, Cambridge, U.K.), with a 8-μl flow cell, was set at 265 nm. The mobile phase consisted of acetonitrile–water–TEAP (396:594:10, v/v/v) and the flow-rate was set at 1.5 ml/min. AMSA concentrations in the samples were determined by measuring the peak-height ratio of the drug, correcting for the recovery of the internal standard and calculating the concentration from a calibration curve. The detection limit was 6 ng/ml at a signal-to-noise ratio of 3:1. The results are expressed in ng/ml for plasma or whole blood and in ng per 10^6 WBCs or RBCs. For comparison of these latter results with those obtained for plasma, it was assumed that $2.5 \cdot 10^9$ WBCs are equal to 1 ml [10,11].

Calibration curve

Samples of 500 μl of pre-treatment plasma were spiked with AMSA. The calibration curve was linear in the range 20–2000 ng/ml with correlation coefficients always exceeding 0.998. The calibration curves of whole blood and WBC suspension were identical to the plasma curves.

Day-to-day variation, intra- and inter-assay recovery

A sample of whole blood was incubated with 2000 ng/ml AMSA for 3 h at 37 °C. With the described dextran procedure the RBCs were sedimented. After centrifugation of the WBC-rich cell suspension, the cell pellet was resuspended in PBS to a cell concentration of approximately $17 \cdot 10^6$ WBC per ml and divided over a number of tubes. These samples were used as cell reference samples. Plasma reference samples, containing 1000 ng/ml AMSA, were prepared as well. The reference samples were stored at −20 °C. For the assessment of the day-to-day variation over the whole procedure of extraction and analysis, reference samples were analysed on eight consecutive occasions.

The recovery of AMSA after extraction was compared with directly injected AMSA in 5% glucose over the range 20–2000 ng/ml. The variations in the recovery within one assay and between the subsequent assays was defined as intra- and inter-assay recovery, respectively.

Assessment of cellular AMSA concentrations

The AMSA concentration in the RBCs ($AMSA_{RBC}$) was indirectly determined, using formula 1 in the Appendix. Since both the number of WBCs and the concentration of AMSA in the WBCs were relatively low, the admixture of WBCs could mostly be neglected in this formula.

The concentration of AMSA in the WBC pellet could be determined directly, but had to be corrected for the amount of AMSA in the remaining supernatant and in the RBCs in the cell pellet, using formula 2.

The volume of the remaining supernatant contains a relatively high concentration of AMSA, which may affect the results considerably. However, this volume can be accurately assessed using formula 3.

RESULTS

Efflux of AMSA during wash steps

Because many commonly used cell separation procedures include dilutions and/or wash steps, the efflux of AMSA during wash steps was examined. All wash steps were performed at 0 °C in order to decrease cellular efflux by inhibition of the cellular metabolism [5]. Purified human WBCs were suspended in plasma to a cell concentration of $29 \cdot 10^6$ WBCs per ml, preincubated for 30 min and incubated with 2000 ng/ml AMSA for 90 min at 37 °C. After 5 and 90 min cellular AMSA concentrations were measured. After 90 min aliquots of 2.5 ml cell suspension were centrifuged and washed with 12.5 ml of either PBS or plasma in order to assess the influence of protein binding. After centrifugation (850 *g*, 10 min) the supernatant was removed. The amount of supernatant remaining was

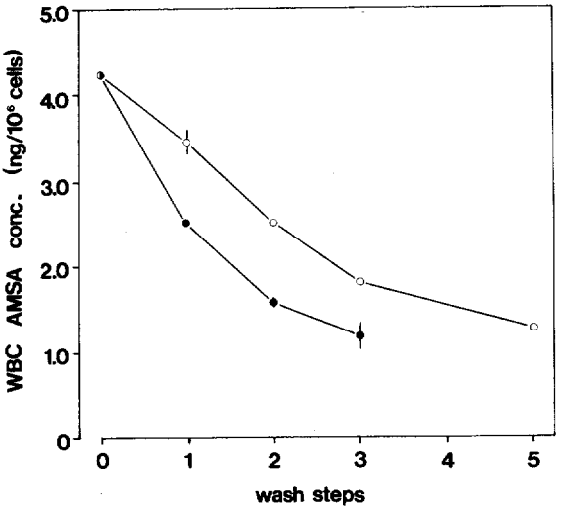

Fig. 1. Influence of wash steps on the cellular concentration of AMSA. Purified WBCs were incubated with 2000 ng/ml AMSA for 90 min at 37 °C and washed one, two, three or five times with (○) PBS or (●) plasma.

determined by weighing the tubes. After resuspension in 0.5 ml PBS, WBCs were counted in two samples, which were stored at −20 °C until extraction. The other cell pellets were washed two, three or five times.

All wash steps were performed on ice. Nevertheless, a rapid efflux of AMSA was observed, with only 42% of the final incubation concentration remaining after three wash steps with PBS (Fig. 1). The efflux in plasma appeared to be even more pronounced with only 28% AMSA remaining after three wash steps. A rapid influx of AMSA into WBCs was also observed. The cellular concentration reached already 74% of the final concentration after 5 min of incubation (not shown).

Influence of dextran upon cellular AMSA concentration

To examine the influence of the addition of dextran on the cellular AMSA concentration, purified WBCs were suspended in plasma to a concentration of $27 \cdot 10^6$ WBCs per ml plasma and incubated for 1 h at 37 °C with 2100 ng/ml AMSA. At the end of the incubation the cell suspension was divided over two tubes. To one tube 1/25 volume of a 25% dextran solution was added. The other tube served as control. After 30 min the upper halves of both suspensions were removed for the determination of cellular AMSA concentrations ($n=4$). The mean cellular AMSA concentration after the addition of dextran was 0.67 ng per 10^6 WBCs (coefficient of variation, C.V. 2.3%). This was only slightly less than for the control (0.74 ng per 10^6 WBCs, C.V. 7.2%). The difference may partly be explained by a dilution of the cell suspension by 4% due to dextran addition.

Enrichment of WBCs after dextran sedimentation of RBCs

The enrichment of WBCs, defined as [concentration $WBC_{cell\ suspension}$/concentration $WBC_{whole\ blood}$] $\times 100\%$ after dextran sedimentation of blood from three

TABLE I

INTRA-ASSAY RECOVERY OF AMSACRINE

Concentration (ng/ml)	n	Recovery (%)		C.V. (%)
		Mean	Range	
2000	8	97.4	93.3–100.1	2.3
1000	8	94.4	92.4–95.4	1.1
500	8	95.1	94.3–95.8	0.6
100	8	93.7	92.1–95.6	1.1
20	2	106.3	103.2–109.4	4.1

leukemia patients and five healthy volunteers, was 132% (range 121–163%). The mean RBC/WBC ratio after dextran sedimentation was 4.4 (range 1.3–8.4).

Linearity of extraction with increasing cell numbers

Purified WBCs were suspended in plasma and incubated for 90 min at 37°C with 2000 ng/ml AMSA. After centrifugation (850 *g*, 10 min), removal of the supernatant and resuspension of the pellet to a cell concentration of $32 \cdot 10^6$ WBCs per ml, duplicate samples were taken containing 10, 15, 30 and $40 \cdot 10^6$ WBCs. A linear correlation ($r = 1.000$) between the number of extracted cells and the extracted amount of AMSA was observed.

Intra- and inter-assay recovery and day-to-day variation

Except for the lowest concentration (20 ng/ml) the mean recoveries of the intra-assay varied from 93.7 to 97.4% with C.V. values of 0.6–2.3% (Table I). The mean inter-assay recoveries ranged from 92.3 to 96.4% with C.V. values of 4.4–7.7% (Table II). The day-to-day variation of the reference plasma and cell samples was 2.3 and 4.7%, respectively.

Chromatography

Chromatograms of a plasma and a cell sample from a patient receiving AMSA are shown in Fig. 2. The dotted lines represent pre-treatment samples.

TABLE II

INTER-ASSAY RECOVERY OF AMSACRINE

Concentration (ng/ml)	n	Recovery (%)		C.V. (%)
		Mean	Range	
2000	7	94.0	82.6–105.8	7.7
1000	8	92.3	88.0–101.3	4.5
500	8	94.7	86.3–100.2	5.1
100	8	96.4	92.2–104.7	4.4
20	7	90.1	64.9–114.7	21.5

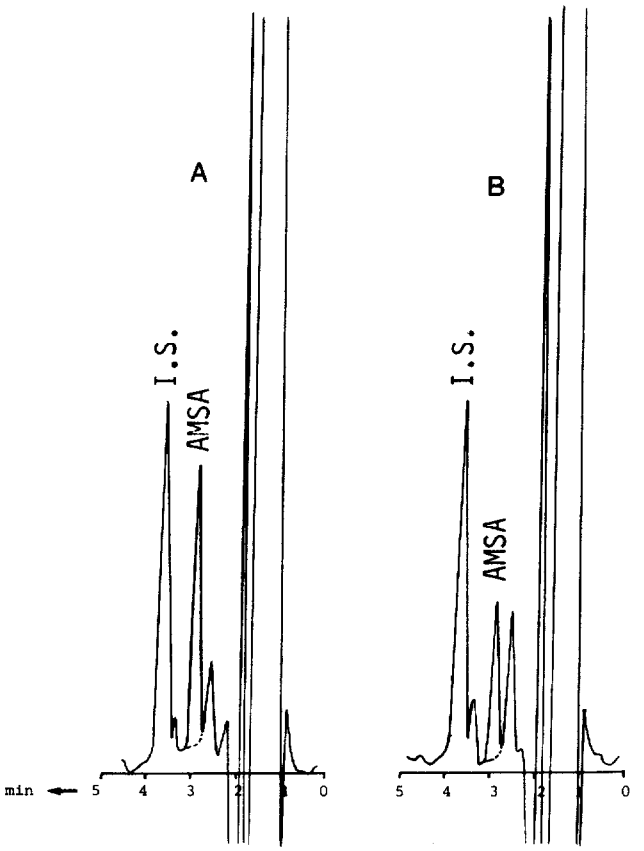

Fig. 2. Chromatograms of plasma (A) and cellular (B) AMSA and internal standard (I.S.) after in vivo administration. The dotted lines represent pre-treatment samples. The extracts of both samples contained 23 and 15 ng AMSA, respectively.

Cellular AMSA concentrations in patients with leukemia

AMSA concentrations in whole blood, plasma, RBCs and WBCs were measured in five patients with acute leukemia to whom AMSA was administered at a dose of 100 mg/m^2 per day during 3 h via a central venous catheter. None of the patients had received AMSA before. No other treatment was given at that time. Blood samples were collected by venipuncture just before the end of the first infusion.

These in vivo data and those of an in vitro experiment, in which blood was incubated with 1000 ng/ml AMSA for 1 h at 37°C, are listed in Table III. The peak concentrations in the plasma of patients varied from 951 to 1735 ng/ml and were consistently higher than the AMSA levels in whole blood (range 737–1531 ng/ml). The concentration of AMSA in RBCs was close to the detection limit of this assay, which may explain the difference between the concentrations in whole blood and in plasma. Cellular AMSA concentrations in WBCs ranged from 1.5 to 4.8 ng per 10^6 cells. The accumulation factor, which is defined as AMSA per ml WBCs/AMSA per ml plasma and based on a mean WBC volume of 400 fl

TABLE III

CELLULAR AMSA CONCENTRATIONS IN FIVE PATIENTS OBSERVED AT THE END OF A 3-h INFUSION OF 100 mg/m^2 AMSA

Patient	Whole blood (ng/ml)	Plasma (ng/ml)	WBCs (ng per 10^6 cells)	RBCs (ng per 10^6 cells)	Accumulation factor*
A	740	966	2.7	0.023	7.0
B	1053	1318	4.8	0.063	9.1
C	1531	1735	1.8	<0.005	2.6
D	737	994	3.9	0.045	9.8
E	875	951	1.5	0.019	3.9
In vitro experiment**	1056	1296	2.1	0.069	4.1

*Accumulation factor = ng per ml WBCs divided by ng per ml plasma.
**Whole blood incubated for 1 h at 37°C with 1000 ng/ml AMSA.

[10,11], is listed in the last column of this table. The mean accumulation factor was 6.5 (range 2.6–9.8). These data are comparable with those obtained from the in vitro experiment.

Plasma and cellular pharmacokinetics

In order to have an indication about the variations of the plasma and cellular AMSA concentration with time, venous blood was drawn from one patient after 0, 15 and 40 min, and after 1, 2, 3.2, 4.2, 8.2 and 19 h after termination of a 3-h infusion of 100 mg/m^2 AMSA. To allow comparison of the plasma and cellular concentrations, the AMSA concentration in the WBCs is expressed in ng/ml, assuming that $2.5 \cdot 10^9$ WBCs equals 1 ml. Each point is the average of two determinations. The mean C.V. values of the WBCs and plasma samples were 1.9% (range 0.1–5.4%) and 2.4% (range 0.0–7.7%), respectively. The peak plasma concentration was 951 ng/ml. The disappearance of plasma was biphasic with a rapid initial phase (half-life, $t_{1/2\,\alpha} = 1.2$ h) and a slower terminal phase ($t_{1/2\,\beta} = 4.6$ h) (Fig. 3). The peak concentration in WBCs was 3750 ng/ml. The concentrations of AMSA in the WBCs correlated well with the concentrations in plasma ($r = 0.967$). The WBC disappearance curve could best be fitted by a $t_{1/2\,\alpha}$ of 0.15 h, a $t_{1/2\,\beta}$ of 2.7 h and a third half-life $t_{1/2\,\gamma}$ of 16.3 h.

DISCUSSION

Pharmacological studies of AMSA in humans have been directed to the kinetics of this drug and its metabolism in body fluids [3,4]. Although certain cells (e.g. leukemic blasts) are the target of the drug, cellular pharmacokinetic data have not been published yet, because no reliable method for its determination was available.

Using radiolabelled AMSA, in vitro studies in mouse leukemia cells [12,7], PY815 mastocytoma cells [5] and HL-60 human leukemia cells [6] showed a rapid efflux of AMSA upon reincubation in a drug-free medium. Since the frequently used methods for the isolation of WBCs from blood or bone marrow include wash steps and/or dilutions, the final cellular AMSA concentrations will

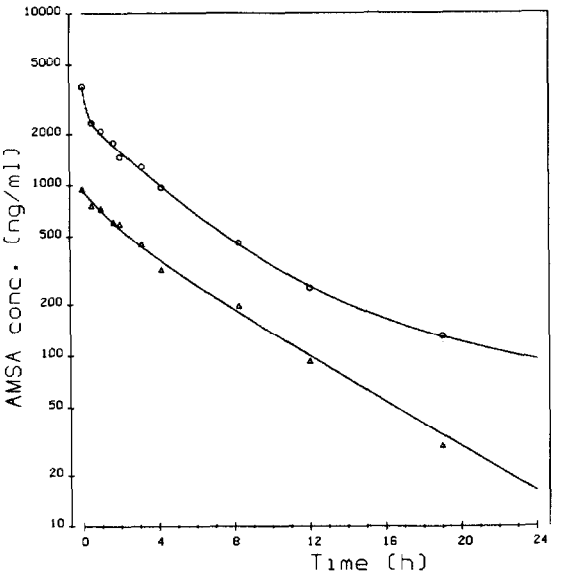

Fig. 3. Disappearance curves of AMSA in plasma and WBCs for patient E, who received 100 mg/m^2 AMSA intravenously over 3 h. Each point is the mean of a duplicate extraction. (△) Plasma concentration, (○) WBC concentration, both expressed in ng/ml.

be considerably affected by these procedures. With all methods tested, including density-cut, gradient and counter-flow centrifugation and specific lysis of RBCs by ammonium chloride, a rapid and considerable efflux of AMSA occurred. This efflux was eliminated using dextran sedimentation of the RBCs and pelletting of the WBCs. Our method also appeared suitable for the determination of cellular AMSA in bone marrow aspirates, provided that the anticoagulant used does not dilute the sample.

Because the AMSA concentration in WBCs is very low, the admixture of small amounts of supernatant in the cell pellet may considerably affect the results. Further improvement of this method is possible by determination of the mean cell volume of the WBCs in each cell suspension in order to reduce the error in the determination of the remaining supernatant.

As compared to adriamycine, another DNA-intercalating drug with a free fraction of 20–30% [13] and an end-infusion accumulation factor of several hundreds [14], AMSA showed much lower cellular concentrations and accumulation factors both in vitro and in vivo. AMSA is bound to plasma proteins to a high extent, resulting in a free fraction of only 3% [15]. The accumulation factor based on the concentration of free AMSA is about 30 times higher than the one based on the total AMSA concentration. To examine the effect of protein binding in the incubation medium on the cellular AMSA concentration, we incubated purified WBCs in plasma as well as in PBS. Cellular drug concentrations in the protein-free medium were 30 to 100 times higher than those in plasma. This suggests that it is especially the concentration of free AMSA that determines the cellular uptake. To compare the results of in vitro studies, protein concentrations in the

media have to be taken into account, along with other factors that influence the extent of protein binding.

The described method is suitable for further studies of the cellular pharmacokinetics of AMSA in man. Such studies have to be extended with clonogenic assays to correlate cellular AMSA concentrations with effectivity.

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APPENDIX

Formula 1

$$AMSA_{RBC} = \frac{AMSA_{Bl} - [(1 - Ht) \cdot AMSA_{Pl}] - (AMSA_{WBC} \cdot WBC_{Bl}/ml)}{RBC_{Bl}/ml}$$

$AMSA_{Bl}$ = concentration of AMSA in whole blood (ng/ml)
$AMSA_{Pl}$ = concentration of AMSA in plasma (ng/ml)
$AMSA_{RBC}$ = concentration of AMSA in the RBCs (ng per 10^6 RBCs)
$AMSA_{WBC}$ = concentration of AMSA in the WBCs (ng per 10^6 WBCs)
Ht = Hematocrit (l/l)
RBC_{Bl} = number RBCs in whole blood (10^6 RBC)
WBC_{Bl} = number WBCs in whole blood (10^6 WBC)

Formula 2

$$AMSA_{WBC} = \frac{AMSA_{Pel} - (AMSA_{Sup} \cdot V_{Sup}) - (AMSA_{RBC} \cdot RBC_{Pel})}{WBC_{Pel}}$$

$AMSA_{Pel}$ = amount of AMSA in the cell pellet (ng)
$AMSA_{Sup}$ = concentration of AMSA in the supernatant (ng/ml)
$AMSA_{WBC}$ = concentration of AMSA in WBCs (ng per 10^6 WBCs)
RBC_{Pel} = number of RBCs in the cell pellet (10^6 WBCs)
V_{Sup} = volume of remaining supernatant in the cell pellet (ml)
WBC_{Pel} = number of WBCs in the cell pellet (10^6 WBCs)

Formula 3

$$V_{Sup} = V_{Pel} - V_{WBC} - V_{RBC}$$

V_{Sup} = volume of remaining supernatant in the cell
V_{Pel} = volume of the cell pellet (determined by weighing the tubes)

V_{RBC} = volume of the RBCs in the cell pellet, based on cell counts and MCV (negligible)

V_{WBC} = volume of the WBCs in the cell pellet (based on cell counts and the assumption that the volume of monocytes, granulocytes and lymfocytes equals 470, 450 and 230 fl, respectively [10,11].

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