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Phase-selective AC adsorptive stripping voltammetric assay for aminopterin and 10-Edam in human serum

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Abstract: Aminopterin was studied as a model compound for its analogues which maintain the pteridine ring in their structure. Its adsorptive behaviour on mercury was studied and the DC adsorptive stripping and phase-selective AC adsorptive stripping conditions were optimized. 10-Edam, an aminopterin analogue, was studied and shown to behave similarly to aminopterin. Phase-selective AC voltammetry provided the best signal and gave a detection limit of 4×10^{-12} M aminopterin in aqueous solution employing an accumulation time of 10 min. The optimized method was applied to the analysis of both aminopterin and 10-Edam respectively in human serum. After extraction with a C_{18} reversed-phase cartridge the detection limit of the method was 1×10^{-8} M aminopterin and the overall assay percentage recovery was 73.5% ($n = 5$) at a concentration of 5×10^{-7} M aminopterin in serum. The analysis of 10-Edam at the same concentration in serum yielded the higher percentage recovery of 94.46% ($n = 5$) following the same procedure.

Keywords: *Aminopterin; 10-Edam; aminopterin analogues; phase-selective AC adsorptive stripping voltammetry; human serum.*

Introduction

Aminopterin is a member of the pteridine family of compounds and was the first antifolate compound to show proven success in the treatment of cancer [1] and in the past has been used widely for the successful treatment of acute leukemia. Even though a highly effective chemotherapeutic drug, it produced unpredictable toxicity at the high doses needed for treatment. Nowadays, it has limited use in cell fusion experiments to select for hybrid cells by killing unfused cells which are deficient in enzymes for nucleotide salvage pathways [2]. However, more importantly the analogues of aminopterin are under constant study [3–8] in an effort to develop more potent and less toxic drugs for the treatment of various cancers including leukemia, lung cancer, and head and neck cancer. Thus, aminopterin analogues, particularly 10-Edam, hold much investigational interest and many are currently undergoing phase 1 and phase 2 clinical trials. The majority of these analogues maintain intact the pteridine ring of aminopterin since substitution at the N_{10} position is one of the main routes to increasing antileukemic effectiveness [9]. Therefore the first electrochemical reduction process (described later), which is the process of analytical importance, will be common to all. The adsorptive stripping voltammetric behaviour of other pteridine compounds has been reported previously by these laboratories [10–13] employing both static mercury drop electrodes and mercury thin film electrodes. Recently in these laboratories a mercury coated carbon fibre ultramicroelectrode was developed for the adsorptive stripping analysis of aminopterin in urine and yielded a sensitive and reproducible method [14]. Few other methods have been reported in the literature for the analysis of aminopterin analogues in biological fluids. A promising approach has been reported however by Tellingen *et al.* [15] using high-performance liquid chromatography with fluorimetric detection for the analysis of 10-Edam. In this present report we studied the parent compound, aminopterin, as a model for the aminopterin analogues bearing in mind that the parent compound and the analogues of major investigational interest have the analytically important reversible reduction process in common. 10-Edam was studied as a typical

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example of the new aminopterin analogues under investigation. Employing a static mercury drop electrode (SMDE) the adsorptive behaviour of aminopterin was studied and compared to that of 10-Edam and the effects of some parameters such as accumulation time, accumulation potential, scan speed, etc. on the preconcentration step were evaluated. The employment of phase-selective AC voltammetry enhanced the sensitivity of the analytical signal in agreement with the theoretical predictions of Laviron [16] for reversible redox couples with both reactant and product strongly adsorbed on the electrode surface. Employment of optimum conditions produced a sensitive method for the analysis of aminopterin and its analogues in human serum. The phase-selective AC adsorptive stripping voltammetric assay was applied to both aminopterin and 10-Edam.

Experimental

Reagents and materials

Aminopterin was purchased from Sigma and used without further purification. 10-Edam (10-ethyl-10-deaza aminopterin) was purchased from Ciba-Geigy (Basle, Switzerland) and used without further purification. Stock solutions of both of 1×10^{-3} M were prepared in 1×10^{-2} M sodium carbonate daily and stored at 4°C in the dark. The initial pH study was carried out using Britton–Robinson constant ionic strength buffer. Ammonium acetate (pH 5) buffer (0.1 M) was prepared by adjusting 0.1 M acetic acid to pH 5 using ammonium hydroxide solution and was used as the background electrolyte throughout the study. All agents were of analytical grade. All solutions were prepared using deionized water obtained by passing distilled water through a Milli-Q (Millipore) water purification system. All deaerations were carried out using purified (N-48) nitrogen (<1 ppm O_2), Sociedad Espanola de Oxigeno. The biological material examined consisted of pools of human serum from healthy individuals. Samples consisted of aliquots of 1 ml of pooled serum spiked with appropriate amounts of analyte to achieve the desired final concentration.

Instrumentation

A Metrohm (Herisau) E-506 Polarecord was used for all voltammetric measurements. Linear potential sweep and cyclic voltammograms were recorded using a Metrohm VA-scanner (E-612) linked to a Linseis XY-recorder (LY1600). A Metrohm EA-290 (Kemula) static mercury drop electrode (SMDE) of drop area 2.2 mm^2 was used as the working electrode for all experiments. All potentials were referred to a Ag–AgCl–KCl, 3 M reference electrode and a platinum wire was used as the counter electrode. A 20 ml electrochemical cell was used which allowed the working electrode, reference electrode, counter electrode and nitrogen delivery tube to be fixed in position through a Plexiglas cover. Solutions were stirred with a constant speed magnetic stirrer (300 rpm). All pH measurements were made using a Crison micropH model 2001 pH meter.

Procedures

Serum sample purification was accomplished by liquid–solid extraction according to a previously reported procedure [11] as follows. Aliquots (1 ml) of serum were spiked with appropriate amounts of the drug to achieve the final desired concentration in serum. The 1 ml aliquot was then diluted to 10 ml with acetate buffer (pH 5) and mixed gently. The resulting solution was passed through a reversed-phase C_{18} cartridge (Sep-Pak, Waters) which was previously activated with 10 ml of pure methanol and 20 ml of water. The effluent was discarded, the cartridge washed with 20 ml of water and the retained materials eluted with 2 ml of methanol. The solvent was evaporated to dryness at 60°C under a stream of inert gas. The dry extract was reconstituted in 20 ml of the background electrolyte (0.1 M ammonium acetate, pH 5) by shaking the tube for 2 min, the contents transferred to the electrochemical cell and purged with oxygen-free nitrogen for 15 min before analysis. A standard addition method was used to quantify the amount of compound present in the sample employing the optimum phase-selective AC voltammetric conditions described later. A serum blank was obtained by following the same procedure using a non-spiked aliquot of serum.

Results and Discussion

Adsorption behaviour/cyclic voltammetry

The adsorption behaviour of aminopterin and its reduction products was initially studied using cyclic voltammetry. The shape of the curves were strongly influenced by the concen-

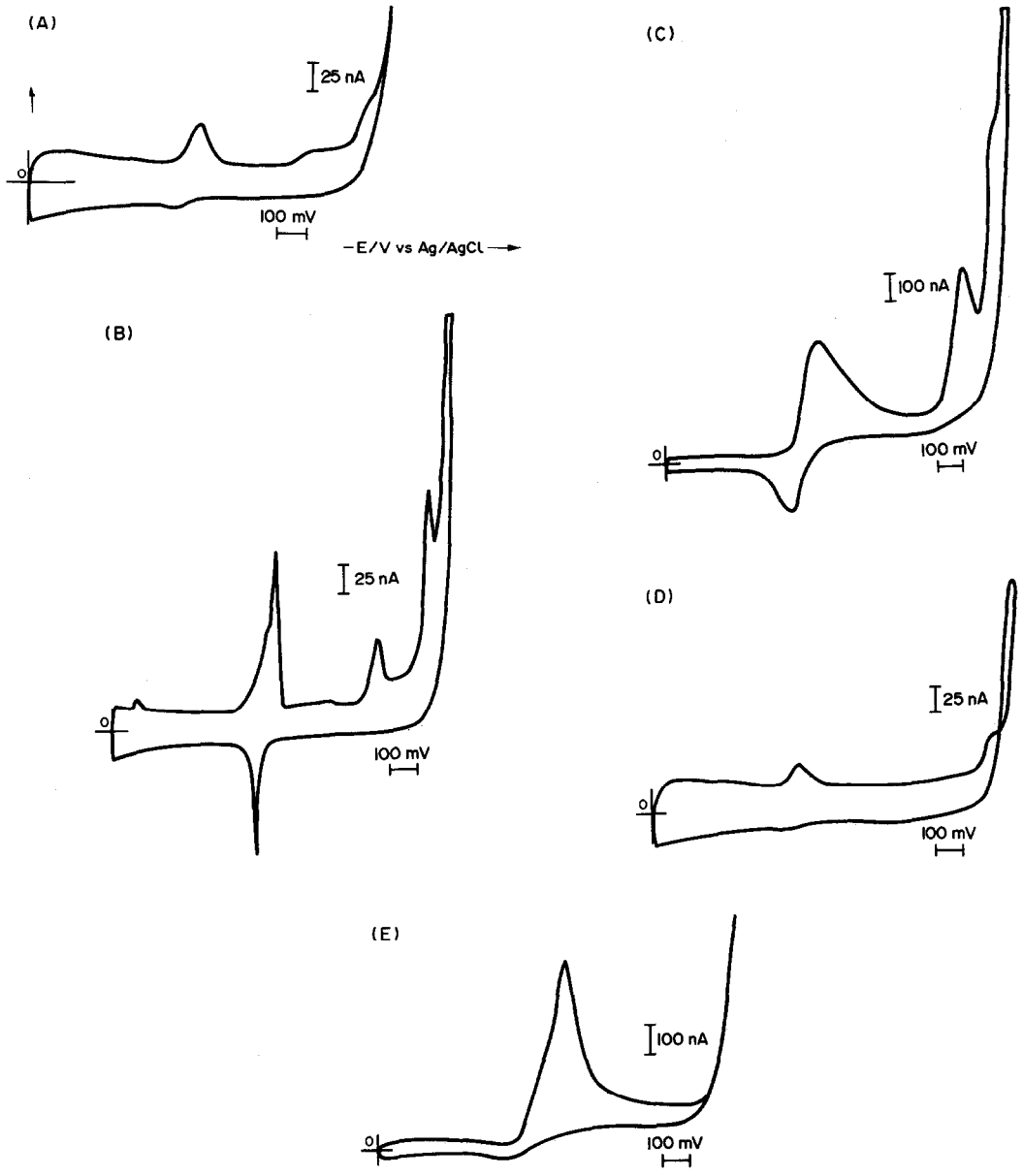

Figure 1
Cyclic voltammograms of aminopterin and 10-Edam aqueous solutions at different concentrations: (A) 1×10^{-6} M AMT, (B) 1×10^{-5} M AMT, (C) 2×10^{-4} M AMT, (D) 1×10^{-6} M 10-Edam, (E) 2×10^{-4} M 10-Edam. Electrolyte = 0.1 M ammonium acetate (pH 5); scan rate = 100 mV s^{-1}.

tration of the species in solution. Figure 1 demonstrates this showing cyclic voltammograms at three different concentrations, (A) 1×10^{-6} M, (B) 1×10^{-5} M, and (C) 2×10^{-4} M of aminopterin. At a concentration of 1×10^{-6} M three symmetrical adsorption controlled reduction processes were observed at -620, -1030 and -1210 mV, respectively. It has been proposed that the first process (1c at -620 mV) is a $2e/2H^+$ reduction of the pteridine ring to yield the 5,8-dihydro derivative. The smaller current of the anodic response (1a at -530 mV) is due to the subsequent tautomerization of the 5,8-dihydroxy derivative to yield the 7,8-dihydroxy derivative. If the direction of the potential scan is reversed directly after the first process the anodic signal increases due to the fact that the majority of the 5,8-dihydro derivative has not yet been tautomerized to the 7,8-dihydro derivative. The second (2c at -1030 mV) and third (3c at -1210 mV) reduction processes are under kinetic control because they are dependent on the chemical rearrangement outlined above.

The second process is due to the $2e/2H^+$ reductive cleavage of the dihydro derivative, produced during the first reduction process, between the C_9 and N_{10} positions to yield R-NH_2 and the 7,8-dihydro derivative of the pteridine moiety. The third electrochemical reduction process is then due to the subsequent $2e/2H^+$ reduction of this 7,8-dihydro derivative to the 5,6,7,8-tetrahydro derivative. The above three reduction processes are seen throughout the concentration range. However, at the higher concentration of 1×10^{-5} M the first process shows a diffusion controlled wave with a superimposed adsorption peak. The second and third reduction processes are under kinetic control because of their dependency on the chemical rearrangement mentioned above and at this concentration remained adsorption controlled. At the highest concentration studied, 2×10^{-4} M, the first wave corresponds to a diffusion controlled process. The second process becomes broader and the third process becomes harder to distinguish from the background discharge. Thus it can be seen that not only aminopterin but also its successive reduction products adsorb onto the mercury electrode. The adsorption of aminopterin onto the electrode surface was also confirmed using medium exchange experiments. The cyclic voltammogram of 10-Edam shows two reduction processes at −530 and −1250 mV, respectively, the first of which is reversible as with aminopterin. The second process exhibited by aminopterin is not seen in the case of 10-Edam since the nitrogen group at position 10 in aminopterin is replaced by a carbon group in 10-Edam. Figure 1(D,E) shows cyclic voltammograms of 1×10^{-6} and 2×10^{-4} M 10-Edam, respectively.

Adsorptive stripping parameters

Using direct current adsorptive stripping voltammetry the pH dependence of the first reduction process, the process of analytical importance, was studied. The peak potential varied linearly with the pH in the range 2–9 with a slope of −60.8 mV pH^{-1} following the equation, E_p (mV) = −60.82 pH −242.23, in close agreement with a $2e/2H^+$ reversible reduction process. A pH of 5 provided the best analytical signal and therefore was used for further studies. The peak current intensity was unaffected by changes in the preconcentration potential in the range 0 to −200 mV. Below −200 mV the signal got worse. Thus, an accumulation potential of −200 mV was chosen for further studies.

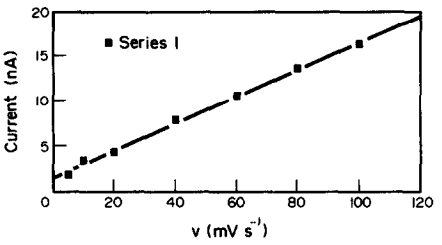

Figure 2
Effect of scan rate (V,mV s^{-1}) on the DC adsorptive stripping voltammetric response for a 1×10^{-7} M aqueous solution of aminopterin. Equation of line: i_p (nA) = −0.1484 V(mV s^{-1}) + 1.5373 (r = 0.9990).

The stripping signal increased linearly with increasing scan rate in the range 5–100 mV s^{-1} (Fig. 2) following the equation: i_p (nA) = −0.1484 V(mV s^{-1}) + 1.5373 (r = 0.9990). A scan rate of 100 mV s^{-1} was used for further direct current adsorptive stripping measurements.

Accumulation curves

Accumulation studies were carried out in stirred solutions using direct current adsorptive stripping voltammetry. A stirrer of fixed rotation speed of 300 rpm was employed. This ensured constant and reproducible convective mass transport to the electrode surface. Accumulation curves were carried out at various different concentrations of aminopterin ranging from 1×10^{-9} to 2×10^{-7} M employing an applied accumulation potential of −200 mV and a constant stirring rate of 300 rpm. After each accumulation period a rest period of 10 s was allowed before the potential scan was started. Figure 3(A) shows the accumulation curves recorded. Accumulation curves of 10-Edam are also shown in Fig. 3(A) for comparison to aminopterin. It can be seen from the accumulation curves that both aminopterin and its analogue, 10-Edam, accumulate in a similar manner even though 10-Edam appears to exhibit a superior rate yielding a steeper slope in the initial section of the curve. As a result of the similar behaviour of both these compounds in terms of their initial reversible reduction process and their accumulative behaviour, aminopterin was taken as a model for this and other similar analogues and used to study further critical parameters both in direct current and alternating current voltammetric modes. The initial portion of each accumulation curve shows a

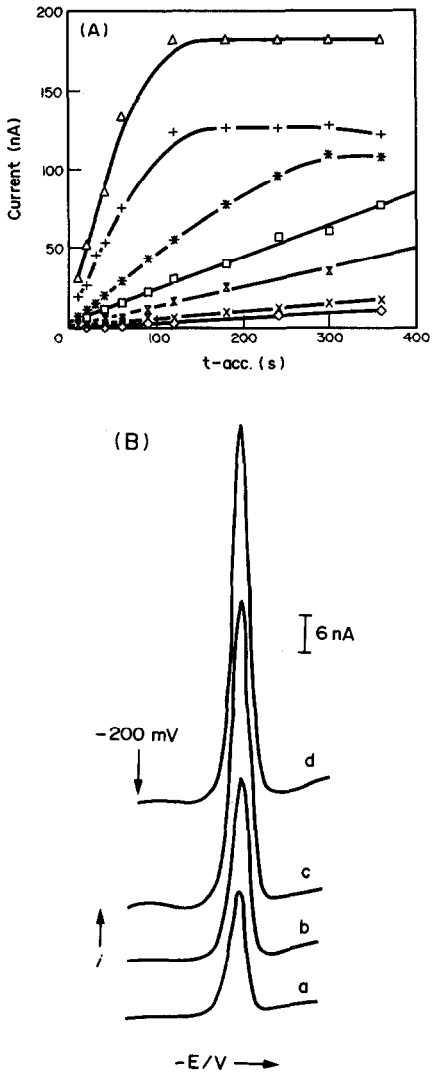

Figure 3
(A) Direct current accumulation curves from agitated, aqueous solutions of various concentrations of aminopterin and 10-Edam. Accumulation potential = 200 mV, scan rate = 100 mV s^{-1}. +, 2 × 10^{-7} M AMT; ★, 8 × 10^{-8} M AMT; □, 4 × 10^{-8} M AMT; ×, 1 × 10^{-8} M AMT; ◇, 1 × 10^{-9} M AMT; △, 2 × 10^{-7} M 10-Edam; ⧖, 2 × 10^{-8} M 10-Edam. (B) Effect of accumulation time on the direct current adsorptive voltammogram of a 2 × 10^{-7} M aqueous aminopterin solution. Accumulation potential = −200 mV; scan rate = 100 mV s^{-1}. Accumulation times: (a) 10, (b) 20, (c) 30 and (d) 40 s.

linear relationship between the intensity of the cathodic stripping peak (first process) and the accumulation time (t_{acc}). The slope growth was faster at higher bulk concentrations of aminopterin. Figure 3(B) illustrates the peak growth with increase in t_{acc} at a concentration of 2 × 10^{-7} M aminopterin. At these higher concentrations the initial linear portion of the accumulation curve is followed by a decrease in slope at a certain surface coverage. Beyond this point the current appears to be practically independent of accumulation time. This surface coverage appears to be the point at which a monolayer of adsorbed compound is present on the electrode surface. Some other pteridines have previously been reported to exhibit hysteresis at this surface coverage resulting in an increased slope [10]. A study of the accumulation curves facilitated the choice of suitable accumulation times for further studies.

Calibration plots

The analytical usefulness of direct current adsorptive stripping voltammetry of aminopterin employing the methodology outlined above was evaluated by studying the linear dynamic ranges and limit of detection achievable. Using an accumulation time of 60 s a linear calibration plot between 1 × 10^{-9} and 4 × 10^{-8} M was yielded with a correlation coefficient (r) of 0.9999 (n = 3) according to the following equation: i(nA) = 4.19 × 10^{8} C_{aminop}/M + 0.837. Employing a lower preconcentration of 20 s a linear range of 1 × 10^{-8} to 1 × 10^{-7} M was achieved following the equation: i(nA) = 1.55 × $10^{8}C_{aminop}/M$ + 0.311 (r = 0.9992, n = 3). Using an accumulation time of 5 min the limit of detection was 4 × 10^{-10} M aminopterin. The reproducibility of the signal was studied at three different concentrations: 5 × 10^{-9}, 1 × 10^{-8} and 5 × 10^{-8} M by employing accumulation times of 180, 60 and 20 s, respectively. At a concentration of 5 × 10^{-9} M a relative standard deviation (%RSD) of 2.46% (n = 10) was calculated. At the higher concentrations of 1 × 10^{-8} M and 5 × 10^{-8} M %RSD values of 3.39% (n = 10) and 4.44% (n = 10), respectively, were yielded. These results seem to indicate that the signal is more reproducible at lower surface coverages. Thus, it would be advantageous to increase the peak intensity without increasing the surface coverage of the electrode. The use of phase-selective AC voltammetry for reversible redox couples with both reactant and product strongly adsorbed has been predicted by Laviron [15] to produce enhanced sensitivity. This enhanced sensitivity would obviously be advantageous for the subsequent analysis of biological samples.

Phase-selective AC studies

Due to the enhanced sensitivity of AC

voltammetry samples containing very low concentrations of analyte may be analysed. A series of studies were carried out to optimize the AC adsorptive stripping voltammetric conditions. Employing the AC1-Tast mode a study of the phase angle (φ) was carried out by measuring the signal characteristics of a 1×10^{-9} M aminopterin solution over the complete range of angles. It was found that at angles close to 90° the current was essentially the charging current component whereas angles closer to 0° produced the best analytical signals. An optimum angle of 9° was chosen since under the experimental conditions employed it was seen to produce the best discrimination of the faradaic current against the capacative current allowing easier measurement of the analytical signal. As the phase angle was increased to angles approaching 90° a dramatic increase in the background current was observed resulting in an increasingly poor analytical signal. The applied AC voltage amplitude (ΔE) was studied under the above conditions and a linear relationship between the cathodic stripping signal and ΔE was observed up to 20 mV according to the following equation: $i(\text{nA}) = 2.133E(\text{mV}) + 0.600$ ($r = 0.9998$). The instrument used worked at a fixed frequency of 75 Hz and did not permit any frequency changes. A scan speed of 10 mV s^{-1} was employed since in AC voltammetry the scan speed does not significantly affect the signal.

Once the optimum conditions were chosen a study of the useful analytical range was carried out. By careful selection of the accumulation time different concentration ranges could be studied. Employing an accumulation time of 5 min linearity was obtained between 1×10^{-11} and 4×10^{-10} M aminopterin according to the following equation: $i(\text{nA}) = 1.70 \times 10^{11}$. $C_{\text{aminop}}/M + 2.615$ ($r = 0.9997$, $n = 2$). Using an accumulation time of 60 s yielded a linear range of 2×10^{-10} to 1×10^{-8} M ($r = 0.9993$, $n = 3$) according to the following equation: $i(\text{nA}) = 3.14 \times 10^{10}$. $C_{\text{aminop}}/M + 18.420$. A limit of detection of 4×10^{-12} M ($S/N = 3$) was obtained when an accumulation time of 10 min was used. A 10-Edam calibration plot was carried out between 1×10^{-10} and 3×10^{-9} M employing an accumulation time of 2 min and showed good linearity according to the following equation: $i(\text{nA}) = 1.07 \times 10^{10}$ $C_{\text{aminop}}/M + 4.549$ ($r = 0.999$, $n = 2$). The reproducibility of the signal was studied at three different concentrations: 5×10^{-10} M ($t_{\text{acc}} = 120$ s), 1×10^{-9} M ($t_{\text{acc}} = 120$ s), and 1×10^{-8} M ($t_{\text{acc}} = 30$ s). The relative standard deviation (%RSD) values calculated were 4.52% ($n = 10$), 2.15% ($n = 10$) and 2.23% ($n = 10$), respectively, which were better than those achieved using direct current adsorptive stripping voltammetry. This may be due to the fact that due to the superior sensitivity of AC voltammetry lower surface coverage is necessary to yield a measurable analytical signal. Thus employing AC voltammetry a sensitive and reproducible method has been developed which may now be applied to the analysis of biological samples (human serum).

Human serum analysis

Taking advantage of the strong adsorption of these compounds on the mercury electrode and the high sensitivity of the phase-selective AC voltammetry the method was applied to the analysis of human serum following the procedure outlined earlier. Serum samples spiked with varying amounts of aminopterin ranging from 5×10^{-6} to 1×10^{-8} M were analysed. Initial studies showed that serum compounds produced two reduction processes at −550 and −970 mV, respectively, the first of which was a broad peak and competed with the analytical signal of interest. Therefore a study of various combinations of open circuit versus applied potential and agitated solution versus quiescent solution accumulation conditions was carried out. It was found that accumulating from a quiescent solution with an applied potential of −200 mV produced the best results and totally avoided the interference problem from serum compounds at −550 mV. Employing relatively short accumulation times was also advantageous in that it discriminated against the large serum compounds which diffuse at a slower rate to the electrode surface than the analyte. Employing these conditions a sensitive and reproducible method was yielded. Using a standard addition method the overall assay recovery was determined at a concentration of 5×10^{-7} M aminopterin in serum (Table 1). The average percentage recovery at this concentration was calculated to be 73.57% ($n = 5$). Figure 4 shows a typical standard addition analysis at this concentration and it clearly demonstrates that no interference is presented by serum compounds under the conditions employed. At the lower concentration of 1×10^{-7} M aminopterin an average

Table 1
Assay recovery

Sample	Added conc. (ng ml^{-1})	Assay result (ng ml^{-1})	% Recovery
1	229.2	171.62	74.88
2	229.2	160.92	70.21
3	229.2	165.65	72.27
4	229.2	174.02	75.92
5	229.2	170.92	74.58

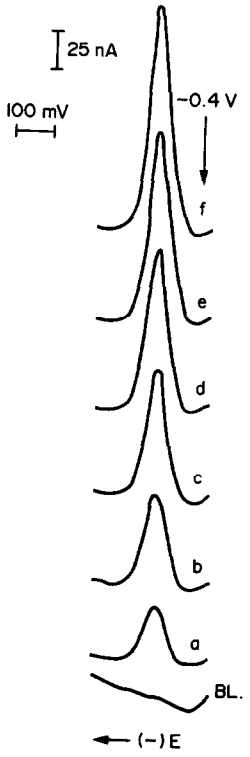

Figure 4
Phase-selective AC stripping voltammograms of a serum blank (BL) and (a) a serum sample extraction (229.2 ng ml^{-1} AMT), followed by standard additions of (b) 20, (c) 40, (d) 60, (e) 80 and (f) 100 µl of a 1×10^{-5} M aminopterin stock solution. Accumulation potential = −200 mV, accumulation time = 10 s. See text for the AC voltammetric conditions and methodology.

percentage recovery of 65.03% ($n = 2$) was calculated and at 5×10^{-8} M aminopterin a percentage recovery of 62.94% ($n = 2$) was yielded. At a concentration of 1×10^{-8} M a signal was yielded which corresponds to the limit of detection ($S/N = 3$) of the method. This method was also applied to 10-Edam analysis in serum (Fig. 5) to prove that it was suitable for the analysis of aminopterin analogues as well as the parent compound. The percentage recovery was evaluated at a concentration of 5×10^{-7} M in serum and yielded

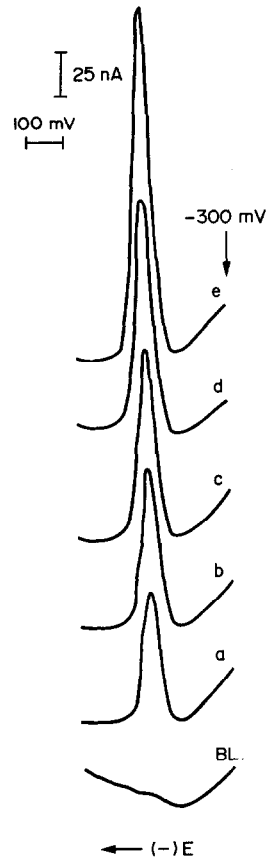

Figure 5
Phase-selective AC stripping voltammograms of a serum blank (BL) and (a) a serum sample extraction (241.2 ng ml^{-1} 10-Edam), followed by standard additions of (b) 10, (c) 20, (d) 40 and (e) 80 µl of a 1×10^{-5} M 10-Edam stock solution. Accumulation potential = 200 mV, accumulation time = 10 s. See text for the AC voltammetric conditions and methodology.

an average of 94.46% ($n = 3$) which is superior to that yielded by aminopterin and may be attributed to the greater retention of 10-Edam on the reversed-phase cartridge due to its structural differences. Thus the method developed is sensitive enough for application to the analysis of clinical samples of patients undergoing chemotherapeutic treatment.

Conclusions

Aminopterin was studied as a model for its analogues which maintain the pteridine ring within their structure. The method developed was applied successfully to the analysis of 10-Edam, an aminopterin analogue of major investigational interest. A sensitive phase-selective AC stripping voltammetric method was developed for the analysis of aminopterin and its analogues in serum. This method is

suitable for the application to clinical analysis of these analogues in the serum of patients undergoing chemotherapeutic treatment. This is an area of major interest since some of these compounds are currently being evaluated as chemotherapeutic agents in phase 1 and phase 2 clinical trials.

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