Journal of Pharmaceutical & Biomedical Analysis
Vol. 10, No. 7, pp. 481–486, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00

Use of derivatization reactions with adsorptive stripping voltammetry for determining fotemustine in biological samples

M.M. DÍAZ DE GUEREÑU, R.J. BARRIO,* A. ARRANZ and J.F. ARRANZ

Department of Analytical Chemistry, Faculty of Pharmacy, University of the Basque Country, Vitoria-Gasteiz, Spain

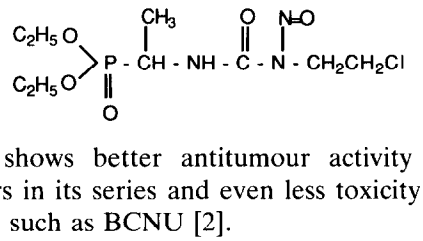
Abstract: A method is described for determining the new fotemustine antineoplastic in human serum. The method is based on the derivatization of the original molecule by means of diazotization and coupling reactions. The derivatization product is determined by means of adsorptive stripping voltammetry. The calibration graphs were linear over the range 6×10^{-9}–8×10^{-7} and 6×10^{-10}–8×10^{-8} M, according to whether the coupling reagent was 1-naphthylamine or 1-naphthol, respectively. At the same time, the detection limits are 4.1×10^{-9} and 1.4×10^{-10} M, respectively. The method was applied to determine this antineoplastic in human serum, after a liquid–liquid extraction process, from which recovery factors of 95.6% has been obtained.

Keywords: *Fotemustine; derivatization; adsorptive stripping voltammetry.*

Introduction

Multifunctional alquilant agents of the antineoplastics group belong to the so-called cytotoxic medicines used in the treatment of cancer. This group of agents was prepared from mustard gas (dichloroethyl sulphide), replacing sulphur in this molecule with an atom of nitrogen, thus reducing the toxicity and increasing its biological activity.

Most medicines classified as alquilant agents contain a structure of ethylenimine or of nitrosourea bis(chloroethyl)-amino [1]. The *N*-(2-chloroethyl) nitrosoureas have high antitumour activity, together with relatively low toxicity. The main members of this group are BCNU (carmustine), CCNU (lomustine) and methyl-CCNU (semustine). A new nitrosourea under study is S10036 or fotemustine. This consists of an aminophosphonic acid group joined to a nitrosourea radical:



It shows better antitumour activity than others in its series and even less toxicity than some such as BCNU [2].

The activity of the nitrosoureas is due to their ability to release active chemical entities. In the case of fotemustine, as for most other nitrosoureas, this release occurs spontaneously in physiological media. Fundamentally, there are two metabolic products whose activity is important [3]: the β-chloroethyl ion, $Cl\text{-}CH_2\text{-}CH_2^+$, a dialkylating species responsible for the formation of lethal cross-links on the DNA molecule, and the isocyanate, R-NCO, responsible for the drug's carbamoylating action.

A range of analytical techniques have been used to determine nitrosoureas. For example, carmustine [4] and lomustine [5] are studied in plasma by means of high-performance liquid chromatography (HPLC) with ultraviolet detection. BCNU and CCNU have been determined by means of gas chromatography, with a nitrogen–phosphorus detector with detection limits of 3 and 1 ng ml^{-1}, respectively [6]. Also by means of GC, and with derivatization with trifluoroacetic anhydride, it is possible to quantify BCNU, CCNU and Me-CCNU in plasma [7]. Although fotemustine is still in its developmental stage, a number of methods for its determination have been published. These include HPLC with UV detection [8] and differential impulse polarography [9], with detection limits of 4.3×10^{-8} and 1.6×10^{-6} M, respectively.

* Author to whom correspondence should be addressed.

In order to lower the relatively high determination and detection limits obtained with electroanalytical techniques such as differential pulse polarography (DPP), chemical derivatization methods have been used. With these methods, it should be possible to achieve two objectives: firstly, for the derivatized substance to have an electrochemical response and secondly, that this substance be adsorbed on the hanging mercury drop electrode (HMDE) in order to be able to use stripping techniques which are much more sensitive than the remaining voltammetries [10]. In this regard, adsorptive stripping voltammetry methods have been developed for determining aromatic amines in which a diazotization reaction is followed by a coupling step with 1-naphthol [11]. A similar method but using different coupling agents has been used for determining synthetic colourants [12], nitrites [13–15], etc.

This paper studies the possibility of determining fotemustine by employing a diazotization reaction in a strong acid medium. The diazonium salt formed may couple with different compounds such as 1-naphthol or 1-naphthylamine in basic medium producing an electroactive compound which can be adsorbed strongly on the hanging mercury drop electrode.

Experimental

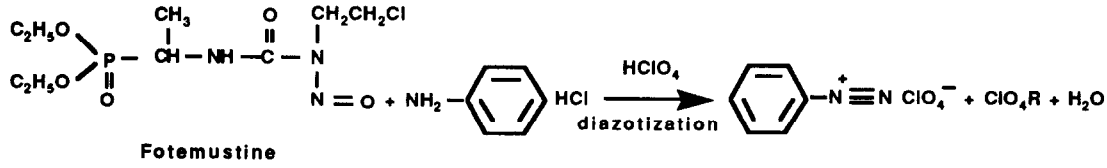
Instrumentation

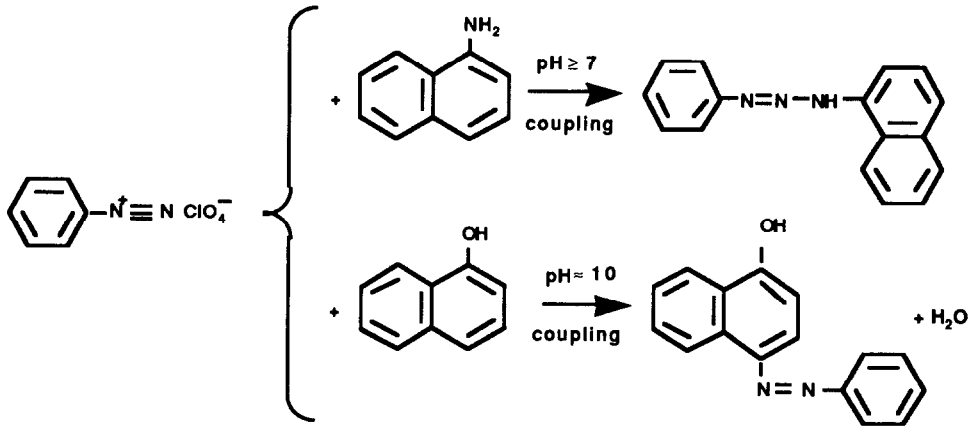
A Metrohm Polarecord E-506 coupled with a Metrohm 663 VA Stand was used. A multimode mercury drop electrode which has a hanging mercury drop electrode (HMDE) was used as the working electrode. The three-electrode system was completed by an Ag–AgCl reference electrode and a platinum auxiliary electrode. A Metrohm VA 612 Scanner and a fast x–y recorder, Linseis LY 1800, were used to obtain the cyclic voltammograms.

A 6302 g Centronic centrifuge (Selecta) was used in the extraction procedure.

Reagents

Fotemustine (diethyl 1-[3-(2-chloroethyl)-3-nitrosoureido] ethyl phosphonate) was supplied by Servier Laboratories (Madrid). Reagent dissolutions of Merck analytical grade were used for the diazotization and coupling reactions: aniline hydrochloride (5 g l^{-1}), perchloric acid (2:1, v/v), 1-naphthylamine (0.05 g dl^{-1}), 1-naphthol (0.3 g dl^{-1}) and acetic acid. The supporting electrolyte was boric-borate buffer at an ionic strength of 0.12 M in KCl. "Milli Q" quality water was used in preparing





where R is: CH_3 CH_2CH_2Cl

$(C_2H_5O)_2$-PO-CH-NH-CO-NH

the dissolutions. Human serum samples were pools from five subjects.

Procedures

Derivatization. 0.5 ml of aniline hydrochloride solution and 3 ml of $HClO_4$ were added to the fotemustine solution, producing the diazotization reaction. After a period (diazotization time), 0.5 ml of coupling reagent (1-naphthylamine or 1-naphthol) is added to the solution. After 5 min the solution is made up to volume of 10 ml. An aliquot (1 ml) of the resultant solution is added to 20 ml of the support electrolyte at the optimum coupling pH. Measurements are taken after allowing an adequate time for coupling to take place.

Adsorptive stripping voltammetry. The solution (20 ml) in the cell was deaerated by passing nitrogen for 10 min. The pre-concentration step was performed by stirring the solution in the electrochemical cell under open circuit conditions at 3000 rpm for a given time period. The stirring was then stopped and, after 15 s a negative-going scan was initiated. The resulting voltammograms were recorded for different combinations of operational parameters.

Fotemustine assay in blood serum. Sample purification was accomplished by liquid–liquid extraction according to the following procedure. A 2 ml volume of serum was added to 10 ml of dichloromethane to cause precipitation of the proteins, which were then separated from the solution by centrifugation at 8000 rpm. The process was repeated three times, grouping the organic phases and subsequently evaporating to low volume in a current of nitrogen. The residue was treated according to the above procedure, in order to determine the fotemustine by means of AdSV-DP. Quantification was achieved by the standard additions method.

Results and Discussion

The cyclic voltammetric study confirmed that the derivatized products Fot-D1 and Fot-D2 are accumulated spontaneously on the hanging mercury drop electrode, under open circuit conditions, from an agitated solution for 4 min. Figure 1 shows the cyclic voltammogram of a 2×10^{-6} solution of Fot-D1 at pH 9.7. A large well-defined cathodic peak can be seen at -0.57 V, which in successive scans is markedly diminished, indicating rapid desorption from the electrode surface.

The spontaneous adsorption of the products Fot-D1 and Fot-D2 on HMDE can be used to determine their voltammetry by using pulse techniques during the scan stage in order to increase sensitivity. The influence of the accumulation time on the analytical signal is shown in Fig. 2. From this figure the saturation

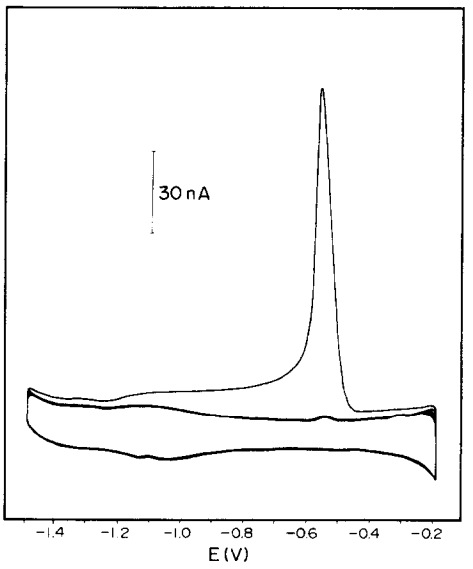


Figure 1
Repetitive cyclic voltammograms at HMDE for 2×10^{-6} M Fot-D1 in boric-borate buffer (pH 9.7), after stirring (3000 rpm) for 240 s at open circuit. Scan rate: 100 mV s^{-1}.

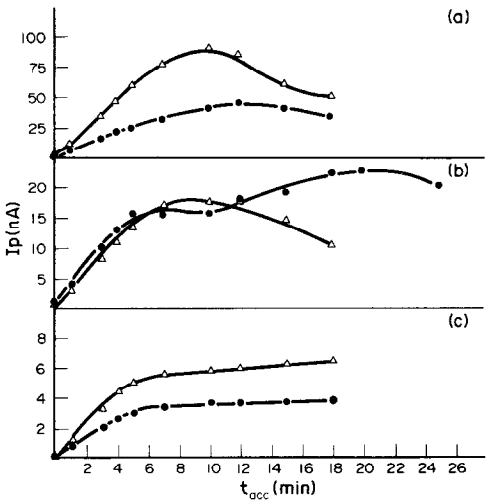


Figure 2
Influence of accumulation time on the peak stripping current. Fot-D1 (△), Fot-D2 (●). (a) 2×10^{-7} mol l^{-1}, (b) 6×10^{-8} mol l^{-1}, (c) 10^{-8} mol l^{-1}. Scan rate: 7.5 mV s^{-1}. Boric-borate buffer (pH 9.7).

times of the electrode surface at each of the concentrations can be deduced. These coincide with the points at which the linearity of the graph is lost. Electroadsorption times greater than those of saturation for the electrode surface cause a loss of linearity on the calibration line [16].

No noticeable influence of the accumulation potential on the peak intensity was observed, as long as this was kept away from the reduction potential of the compound. For this reason, −0.2 V vs Ag–AgCl was selected as being optimum.

The effect of the pH on the intensity and peak potential can be observed in Fig. 3. Working within a pH range of between 7 and 13, sufficient to provoke the coupling reaction, a variation in the i_p was observed, the maximum value for which appears at pH 9.7 for Fot-D1 and at pH 10.0 for Fot-D2. On the other hand, the increase in pH provoked a movement of the peak potential towards more negative values.

Given that two reactions take place in the derivatization process, it was necessary to optimize each of the reaction times. Figure 4

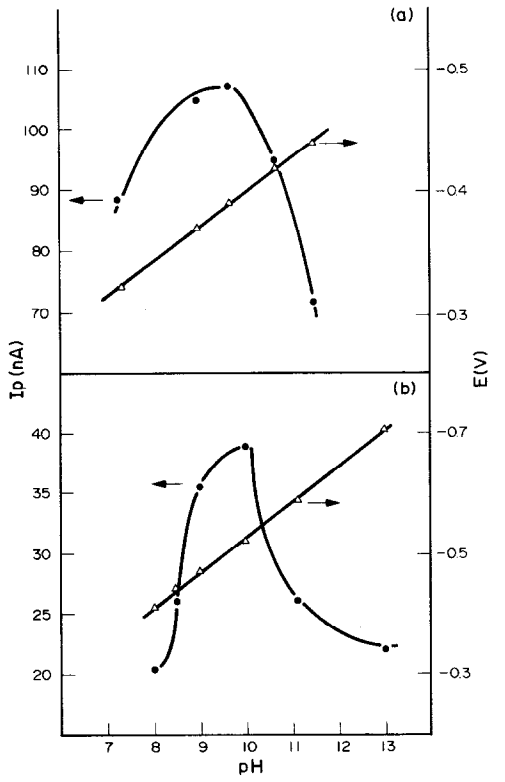


Figure 3
Influence of pH on the intensity (●) and peak potential (△). (a) Fot-D1, (b) Fot-D2. Other conditions as in Fig. 2. Fotemustine concentration: 2×10^{-7} mol l^{-1}.

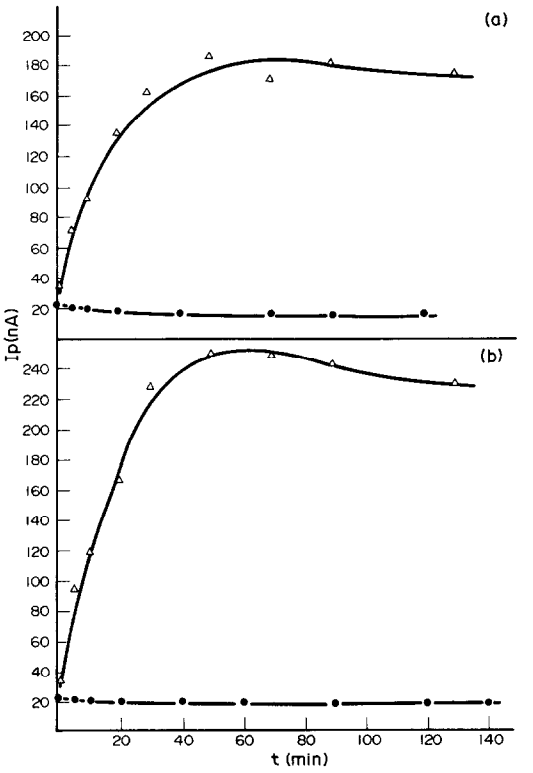


Figure 4
Effect of diazotization (△) and coupling (●) time on the stripping voltammograms. (a) Fot-D1, (b) Fot-D2. Other conditions as in Fig. 2. Fotemustine concentration: 2×10^{-7} mol l^{-1}.

shows the influence of the diazotization time. For both derivatized substances, intensity increases with a steep slope to 50 min at which it remained practically constant. In order to reduce the analysis time, but still maintain good detection limits, a diazotization time of 5 min, which must be perfectly controlled, was chosen. In Fig. 4, it can be seen that the coupling time has virtually no influence on the analytical signal.

Optimized instrumental and dissolution parameters for each of the derivatized forms studied are summarized in Table 1.

Under these conditions the calibration lines were obtained:

$$i_p = 15.7 + 6.3 \times 10^8\, C \quad (r = 0.9963)$$
for Fot-D1

$$i_p = 1.29 + 9.9 \times 10^8\, C \quad (r = 0.9952)$$
for Fot-D2,

where i_p is expressed in nA and C is the initial concentration of fotemustine in mol l^{-1}. The linearity ranges are 6×10^{-9}–8×10^{-7} M and 6×10^{-10}–8×10^{-8} M, respectively.

Table 1
Optimized instrumental and solution parameters

	Fot-D1	Fot-D2
Pulse amplitude	100 mV	80 mV
Drop size	0.52 mm^2	0.52 mm^2
Scan rate	7.5 mV s^{-1}	7.5 mV s^{-1}
Stirring speed	3000 rpm	3000 rpm
Accumulation time	300 s	300 s
Rest time	15 s	15 s
pH	9.70	10.00
Buffer	H_3BO_3/BO_3^-	H_3BO_3/BO_3^-
Ionic strength	0.12 mol l^{-1}	0.12 mol l^{-1}
Aniline hydrochloride	0.5 ml (5 g l^{-1})	0.5 ml (5 g l^{-1})
Perchloric acid	3 ml (2:1)	3 ml (2:1)
Coupling reagent	1-naphthylamine 0.5 ml (0.05 g dl^{-1})	1-naphthol 0.5 ml (0.3 g dl^{-1})
Diazotization time	5 min	5 min
Coupling time	5 min	5 min

The reproducibility of the method was determined from 10 replicate reactions on fotemustine at a concentration of 1×10^{-8} M. The relative standard deviations (RSD) were 4.2 and 3.6% for Fot-D1 and Fot-D2, respectively.

Detection and determination limits have been estimated using criteria set by Caulcutt and Boddy [17]. Thus, when the coupling reagent is 1-naphthylamine, the standard deviation of the blank is 0.2 nA thereby establishing a detection limit of 4.1×10^{-9} M and a determination limit of 5.1×10^{-9} M. If the coupling reagent is 1-naphthol, the standard deviation of the blanks is 0.1 nA and the corresponding detection and determination limits are 1.4×10^{-10} and 6.1×10^{-10} M, respectively.

Application to the analysis of human serum

Figure 4 shows the results obtained by applying the standard additions method and following the method described above. In this case, 1-naphthol was chosen as coupling agent.

Using the liquid–liquid extraction method, a mean recovery of 95.6% from spiked serum samples was obtained, in the concentration range of 1×10^{-8} M. The reproducibility of the method was determined by successive measurements on 10 7×10^{-8} M (22.1 ng ml^{-1}) solutions of fotemustine in serum. The average peak current was 1.3 nA (range 1.4–1.5 nA) and the relative standard deviation was 5.0%. The high mean recovery obtained at low concentrations permitted the establishment of detection and determination limits for fotemustine in serum of 4.4 and 19.3 ng ml^{-1} using 1-naphthol as coupling agent.

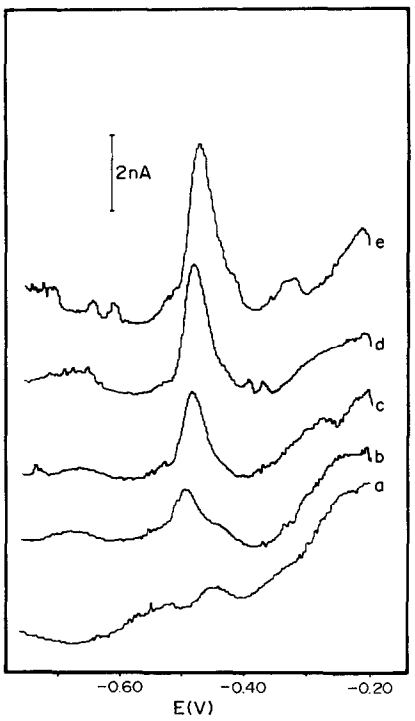


Figure 5
Voltammetric curves (AdSV-DP) obtained for the determination of fotemustine extracted from human serum. Coupling agent: 1-naphthol. (a) blank, (b) serum spiked with fotemustine 10^{-7} M, (c, d, e) standard additions of 20 µl of 10^{-6} M of Fot-D1 (at 20 ml of boric-borate buffer).

If it should be necessary to considerably lower the value of the determination limit, this can be achieved simply by increasing the diazotization time. In this way concentrations of 10^{-9} M have been determined in serum by raising the diazotization time to 30 min.

There may be interferences, above all due to the complexity of the serum matrix and the appearance of fotemustine metabolites. Neither of the two above mentioned metabolites, β-chloroethyl and isocyanate, has —N=O groups and will therefore not produce a derivative using the proposed method. Nevertheless the isocyanate group may become electrochemically active under these experimental conditions. That is why there may appear small peaks at very negative potentials, −1.2 V, when the fotemustine has been in the serum for more than 15 days. At such negative potentials the interference with the analytic signal arising from fotemustine is negligible.

The presence of other surfactive compounds which might affect the response was investigated, particularly those which might be present in serum samples. The effects of albumin and gelatin were studied with 10^{-7} M fotemustine solution. The addition of 2 mg l^{-1} of albumin and 2 mg l^{-1} of gelatin give rise to a Fot-D2 peak depression of 52.3 and 59.4%, respectively. Thus the use of the liquid–liquid extraction procedure proposed above and the standard additions method for the elimination of the matrix effect are both necessary.

Conclusions

A sensitive and selective differential pulse adsorptive stripping voltammetry method for determining the antineoplastic fotemustine has been presented. The limit of detection reached (4.0×10^{-10} M) in aqueous medium was two orders of magnitude lower than those reached with HPLC–UV techniques (4.3×10^{-8} M). Before determination, fotemustine was subject to a diazotization reaction, the product of which was subsequently subject to a coupling reaction. Two coupling reagents were used: 1-naphthol and 1-naphthylamine. The method had a lower limit of detection if the first was chosen. However, for concentrations of around 10^{-7} M, it was preferable to use the second, as linearity was maintained.

Acknowledgements — The authors wish to express their thanks to Laboratorios Servier (Spain) for their financial assistance and the interest and trust shown with the supply of the active fotemustine product.

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[Received for review 23 September 1991; revised manuscript received 28 November 1991]