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Short Communication

Fluorimetric determination of tauromustine (a novel antitumour agent) in formulations and biological fluids

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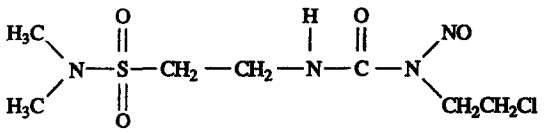
Introduction

Tauromustine, 1-(2-chloroethyl)-3-[2-(dimethylaminosulphonyl)ethyl] 1-nitrosourea (TM) (Scheme 1), is a novel antitumour agent based on the endogenous aminoethanesulphonic acid, taurine. Preclinical studies with tauromustine have revealed its potent antitumour activity against several experimental tumours *in vivo* and *in vitro* [1, 2] and its efficacy against nitrosourea-resistant tumours [3].

An HPLC method has been described for the determination of tauromustine and its metabolites in plasma and urine [4, 5]. However, this method needs a sophisticated and expensive instrument. Recently, a polarographic method has been developed for the determination of tauromustine in tablets [6]. This method is also tedious and suffers from the general limitations of the polarographic technique. It is evident therefore that there is still a need for a simple, sensitive and reliable method for the determination of tauromustine especially in biological fluids. Fluorimetry by virtue of its high sensitivity was the method of choice to meet such requirements.



Scheme 1

Experimental

Apparatus

An Aminco Bowman spectrofluorimeter, model j 4-8960 was used. The activation slit control was set at 5 mm at full sensitivity. The measurements were performed using a 1-cm quartz cell.

Materials

Tauromustine and its tablets (20 and 50 mg) were provided by Pharmacia, Leo (AB Helsingborg, Sweden). Fluorescamine was purchased from Aldrich (WI, USA). All solutions were prepared in spectroscopic grade solvents. Acetone (BDH) and distilled water were checked before use for the presence of fluorescent contaminants.

Solutions

A stock solution of tauromustine (1 mg ml^{-1}) was prepared in distilled water and diluted with the same solvent as appropriate. Fluorescamine reagent (10 mg in 100 ml) was prepared in acetone; the solution was kept in refrigerator. A solution was prepared freshly each week. Borate buffer (pH 7.2), phosphate buffer (pH 6) and Britton Robinson buffer (pH 12) were used.

Procedure

Construction of calibration graph. A stock solution containing 1 mg ml^{-1} of tauromustine was prepared in Britton Robinson buffer (pH

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12) by ultrasonication for 15 min. The solution was further serially diluted with water to a final concentration of 25 μg ml^{-1}. Aliquots of the final solution were transferred to a series of 10-ml measuring flasks to produce solutions in the concentration range 0.25–4.5 μg ml^{-1}; then 5 ml of borate buffer (pH 7.2) was added to each solution. After mixing well, 1 ml of fluorescamine solution was added and the solution was diluted to 10 ml with borate buffer; the fluorescence intensity was measured at 472 nm using an excitation wavelength of 370 nm. The percentage relative intensity was plotted against the final concentration of tauromustine to obtain the calibration graph.

Procedure for the tablets. Twenty tablets (20 and 50 mg) were weighed and powdered. An accurately weighed amount of the powder equivalent to 50 mg of the drug was extracted with 30-ml portions of water, filtered into a 100-ml measuring flask and diluted to 100 ml with water; this solution was treated as described under construction of the calibration graph.

Procedure for biological fluids. The plasma or urine sample was acidified with 2 M hydrochloric acid (10 μl per ml of plasma or urine), tauromustine solution was added and the solution was thoroughly mixed in a vortex mixer for 15 min and diluted to 25 ml in a measuring flask with phosphate buffer (pH 6). The mixture was mixed in a vortex mixer and extracted with three 10-ml portions of hexane–ethyl acetate (40:60, v/v). The extract was evaporated under a gentle stream of nitrogen at room temperature. The residue was dissolved in distilled water and the solution was treated as described under construction of the calibration graph.

Calculation. The nominal content of the drug was calculated either from a previously plotted calibration graph or from a linear regression equation.

Results and Discussion

Generally, the stability of the chloroethyl nitroso urea is pH-dependent, the drug showing poor stability under alkaline conditions [7]. Based on this observation tauromustine, which has an amide functional group, was found to be hydrolysed easily in Britton Robinson buffer (pH 12). TLC study of the alkaline hydrolysate gave two spots on a TLC plate using silica gel G as the adsorbent and 96% methanol–25% ammonia [8:2, v/v] as the mobile phase. One of the two spots (R_f = 0.64) gave a violet-red colour with ninhydrin spray after heating for 15 min at 105°C indicating the presence of a primary amino functional group. The amine produced was allowed to react with fluorescamine in borate buffer (pH 7.2) forming a highly fluorescent derivative. In a fraction of a second at room temperature the reaction is complete; in less than 1 min the excess reagent is destroyed and the fluorophore is stable for several hours [8]. At the wavelength of maximum excitation and emission neither the drug nor fluorescamine fluoresces. Figure 1 shows the fluorescence spectra of the fluorophore. The suggested reaction pathway is shown in Scheme 2.

The resulting fluorescence is proportional to tauromustine concentration over the concentration range 0.25–4.5 μg ml^{-1} with a lower limit of detection (S/N = 2) of 1 ng ml^{-1}. For this range, linear regression analysis of the plot of tauromustine concentration and the measured fluorescence gives the following equation:

$$C = 0.0074 + 0.0495\,F \qquad (r = 0.9998),$$

where C is the concentration of the drug in μg ml^{-1}, F is the percentage relative intensity and r is the correlation coefficient. To test its validity, the method was applied to the determination of authentic samples of tauromustine.

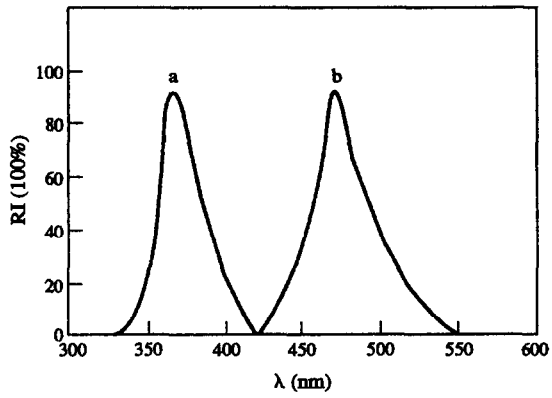
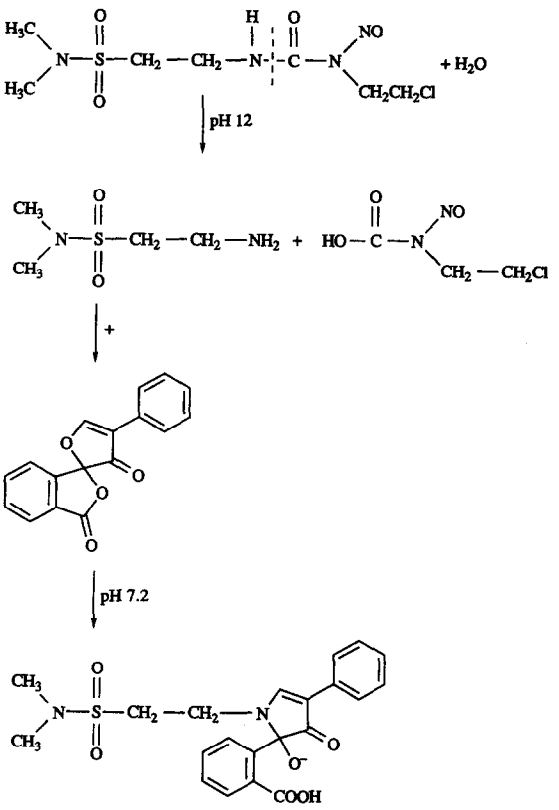


Figure 1
Fluorescence spectrum of tauromustine fluorescamine reaction product (4.5 μg ml^{-1}). a, Excitation spectrum; b, emission spectrum.



Scheme 2
Proposed reaction pathway between fluorescamine and the hydrolytic product of tauromustine.

Table 1
Performance data for the fluorimetric determination of tauromustine with fluorescamine

μg Taken	μg Found	% Recovery
0.2	0.199	99.5
0.5	0.499	99.98
0.8	0.799	99.97
1.0	0.999	98.98
2.0	1.979	98.95
3.0	3.024	100.81
4.0	4.049	101.22
4.5	4.524	100.53
Mean		100.10
SD		0.679

Table 2
Application of the proposed method to the analysis of tauromustine tablets

	% Recovery ± SD*	
Preparation	Proposed method	Reference method [6]
Tauromustine tablets (20 mg)	100.24 ± 0.63 t = 0.488 F = 9.482	99.89 ± 1.94
Tauromustine tablets (50 mg)	100.04 ± 0.67 t = 1.022 F = 29.192	100.66 ± 3.62

*The results are the mean of eight separate determinations.
Tabulated t (P = 0.5) = 2.11 (17 degrees of freedom).
Tabulated F (P = 0.5) = 3.14 (10 and 7 degrees of freedom).

The results summarized in Table 1 show that the proposed method is accurate and precise. To check the stability of tauromustine, the sample was reacted directly with fluorescamine at pH 7.2. The same concentration was hydrolysed as described above and then reacted with fluorescamine. The difference of the two readings corresponds to the intact tauromustine.

The proposed method was further applied to the determination of tauromustine in tablets. The results in Table 2 are in accordance with those obtained with the reference method [6]. Tablet excipients such as talc, starch, lactose, gum and magnesium stearate did not interfere with the assay but gelatin interfered to give high results. From the results in Table 2 the proposed method is more accurate and precise than the reference method [6].

The high sensitivity achieved by this method renders it applicable for the determination of the drug in biological fluids (Table 3) where

Table 3
Application of the proposed method to the determination of tauromustine in urine and plasma

	Urine			Plasma		
1	μg Added	μg Found	% Recovery	μg Added	μg Found	% Recovery
1	0.2	0.175	87.5	0.2	0.175	87.5
2	0.5	0.475	95.0	0.5	0.45	90.0
3	0.8	0.747	93.3	0.8	0.727	90.9
4	1.0	0.925	92.5	1.0	0.9	90.0
5	2.0	1.897	94.87	2.0	1.85	92.5
6	3.0	2.851	95.04	3.0	2.801	93.38
7	4.0	3.802	95.00	4.0	3.728	93.2
8	4.5	4.251	94.47	4.5	4.176	92.81
Mean			93.46			91.28
SD			2.41			2.05

the preliminary stability studies in aqueous media and plasma *in vitro* at various pH values have indicated that tauromustine, like other nitrosourea compounds shows maximum stability at lower pH values [4]. The plasma was therefore acidified first; the pH of the plasma was then raised by addition of buffer (pH 6) before extraction with organic solvents. This procedure minimized interference from other endogenous plasma substances [8]. Samples were extracted according to the procedure described above. The mean absolute recovery was 91.28 ± 2.04% for plasma samples and 93.42 ± 2.41% for urine samples; these results demonstrate that the recoveries were not influenced by the plasma matrix.

Detection limit

Initially, the detection limit was estimated from a signal-to-noise ratio of 2. In order to obtain more exact values, aqueous solutions containing tauromustine in concentrations near the estimated detection limit were prepared. The detection limit was 1 ng ml^{-1} for tauromustine.

Conclusions

A highly sensitive, selective and rapid fluorimetric method for the determination of tauromustine has been developed. The method is based on alkaline hydrolysis of the drug followed by reaction with fluorescamine at pH 7.2 to produce a highly fluorescent probe. The various experimental parameters affecting the development of fluorophore were carefully studied and the optimum conditions were incorporated into the procedure. A proposal of the reaction pathway was presented. Under the described conditions, the proposed method is applicable over the concentration range 0.25–4.5 μg ml^{-1} with a lower limit of detection ($S/N = 2$) of 1 ng ml^{-1}. The suggested method was further applied to the determination of tauromustine in tablets and in urine and plasma. The results agreed statistically with those obtained with a reference method. The method is characterized by being highly sensitive, simple and rapid. The method is also stability-indicating.

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