An HPLC method for the determination of busulfan in biological samples

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ABSTRACT

An HPLC analytical method for assessing plasma busulfan rat has been developed. This method uses 1,5-Bis (metansulfoniloxy) pentane, as internal standard, dimethylformamide (DMF) as solvent, Tri Butyl n Phosphine as an antioxidant and 8 Mercaptoquinoline (Q) as a reagent. We have evaluated the accuracy, reproducibility and speed, with satisfactory results.
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INTRODUCTION

A review of the literature highlights the lack of available analytical methods for the assessment of busulfan in biological samples. A remarkable feature of all is that they are based on chromatography, liquid or gaseous. Thus, gas chromatography mass spectrometry has been used, which is a very specific technique, but little sensible[1-3]. Gas chromatography with electron capture detection is more sensitive, with a detection limit of 0.01 mg/ml, but with the disadvantage of some interferences[4,5].

These two methods, based on gas chromatography are tedious, as there are to get derivatizing the compound and make it volatile. This has motivated the development of methods based on high resolution liquid chromatography, with ultraviolet detection[6-9] or by masas spectrometry[10]. While the first is simple and economical, has the disadvantage of being insensitive. The second presents a detection limit of 50 µg/ml and does not require derivatization of busulfan. However, it is not without its drawbacks, as its high cost makes it inaccessible for the vast majority of cases.

As is clear from the discussion up here, is the development of new analytical methods for determining levels of busulfan, which are easy to use, reproducible and economic.

The analytical method used in this report for the assessment of busulfan in plasma samples has been developed and perfected by our research group. The method is based on liquid chromatography with fluorimetric detection and allows adequate separation of busulfan of the remaining components of the plasma, being a sensitive, rapid and specific. The sample preparation is relatively simple and requires only small sample volumes.

For the quantification of antineoplastic present in plasma samples, we used high performance liquid chromatography (HPLC), according to the method described below.

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MATERIAL AND METHODS

Elements and chromatographic conditions

It has been used a liquid chromatograph, high performance liquid 'Perkin Elmer' consists of the following modules:

- Pump Perkin-Elmer, Series 10.
- Autosampler Perkin-Elmer ISS200 Advanced LC Sample Processor
- Waters fluorescence detector, model 420 BC. We used an excitation filter of 360 nm and an emission filter of 425 nm
- Recorder-integrator, Perkin-Elmer Nelson, model 1020.

The chromatographic system used consists of a stationary phase and a mobile phase.

As stationary phase was used a commercial reverse phase column C 18 Nova-Pak ®″, 15 cm long and 3.9 mm internal diameter.

It has also used a precolumn 'Tecnokroma' model C 135 B, with two filters of 2 mm and a filling pore film C18 with a particle size of 40 micrometers. Its presence extends the life of the column, as it prevents the passage of any solid contaminants.

The mobile phase consisted of a mixture of acetate buffer pH = 5 and acetonitrile, in volumetric proportions 45:55. The flow was maintained at 1 ml / min.

Under the conditions described, the peak of busulfan and internal standard in the chromatogram are separated from other plasma components, as shown in figure 1.

Sample preparation and quantification of busulfan

The material used for this has been:

- An agitator 'Vortex' model 'D 051'.
- A refrigerated centrifuge model Sigma 2K15 with a rotor No. 12 145. The speed at which the samples were centrifuged 10 000 g or 11,500 rpm.
- A thermostatic bath 'P Selecta' used at a temperature of 80°C.

The products and reagents used

(1) Internal standard

We used 1.5 Bis (metansulfoniloxy) pentane, and was summarized as follows: A metansulfonyl chloride solution (2.52 g, 0.022 mol) in methylene chloride (4 ml) was added slowly a solution of 1,5-pentanediol (1.04 g, 0.01 mol), pyridine (1.74 g, 0.022 mol) and methylene chloride (4 ml) while stirring at 0°C. The mixture was stirred at 25°C for one hour and extracted with water (7 ml) four times. Methylene chloride was evaporated at 55-65°C until a viscous liquid which was added 5 ml of ethanol, stirred and introduced in a freezer (about 10°C) for the separation of both phases. The ethanol phase was discarded and the product was washed again with 5 ml of ethanol. After heating at 70°C to remove residual ethanol, we obtained a viscous liquid which was identified as 1,5-Bis (metansulfoniloxy) pentane by NMR.

For the assessment of the biological samples was used diluted 1/50451 of the internal standard in N, N dimethylformamide (DMF).

(2) Antioxidant

TBF 10%: 0.1 ml of Tri Butyl n Phosphine ($C_{12}H_{27}P$, Sigma ®) and 0.9 ml of DMF.

TBF 1%: 0.1 ml of 10% TBF and 0.9 ml of DMF.

(3) Reagent

Q: 10 mg of 8 Mercaptoquinolina (Thiooxiline, Sigma ®) in 1 ml of DMF.

For the assessment of plasma samples previously placed 10 µl of DMF (to have same volume as in the patterns) in a tube 'eppendorf', to which were added 0.1 ml of plasma and shaken manually for a few seconds. Then were added 20 µl of the dilution of internal standard and 0.1 ml of acetonitrile. After agitation in a 'Vortex Mixer', the mixture was centrifuged at 10000 g for 5 minutes, 0.150 ml decanting the supernatant into another tube 'eppendorf'. In it, he added 10 µl of TBF at 1%, 10 µl of Q and 10 µl of 0.1 N NaOH. This mixture was stirred and incubated in a water bath at a temperature of 80°C for 1 hour. Samples prepared were frozen until injection into the chromatograph.

For the quantification of busulfan in the samples, standards of known concentration were treated in the same way as described for the samples.

(4) Evaluation of the analytical method

To test the linearity of method, patterns of busulfan were prepared in rat plasma in a field of concentrations of 50-30000 µg/ml, as if they were unknowns and, after

TABLE 1 : Precision and accuracy of the chromatographic method used for the evaluation of busulfan in plasma samples. The first column shows the actual concentrations of busulfan in the patterns (Creal) and the following presents the mean values (Ccalculada, n = 7), the percentage of deviation of Ccalculada about Creal (deviation (%)) and coefficient of variation (CV) of the analytical method

C_{real} (ng/ml)	C_{calc} (ng/ml)	Desviación (%)	C.V. (%)
200	190	5	8
2000	1883	6	5
5000	4606	8	5

Figure 2 : Plot of calculated values of busulfan concentration in plasma versus the real concentration
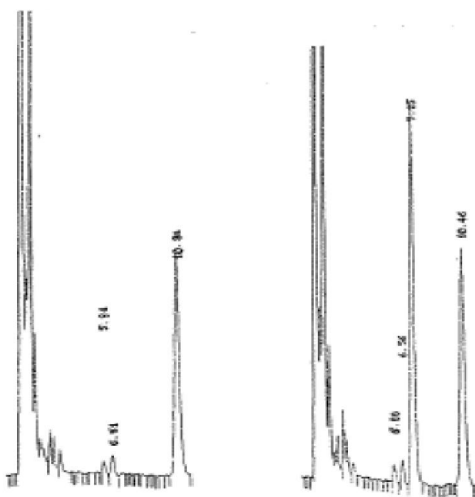

Figure 1 : To the left of this figure is represented the chromatogram obtained from a plasma sample containing no busulfan. The peak of 10.34 minutes corresponds to that arising from the internal standard. To the right is represented the chromatogram obtained from a plasma sample containing busulfan. The peak of 7.25 minutes for the derivative of the antineoplastic and the peak of 10.46 minutes for the internal standard derivative

Figure 3 : Calibration line obtained for busulfan in plasma. It has represented the ratio between the area of busulfan and the area of internal standard (R) vs. concentration (C) of busulfan. In all tests, the calibration lines had correlation coefficients exceeding 0.999

injection into the chromatograph, were obtained the corresponding peaks. The ratios between the peak area corresponding to busulfan and the corresponding peak area internal standard showed excellent linearity in terms of the concentration of busulfan.

In order to judge the accuracy and precision (reproducibility) of analytical method, we proceeded to assess patterns of busulfan in rat plasma, concentration 200, 2000 and 5000 µg/ml, treating them the same way as described for samples . The drug concentrations were obtained for each pattern, after evaluating seven times, was averaged and the coefficient of variation. The difference between the average value and the theoretical concentration (real) of busulfan for
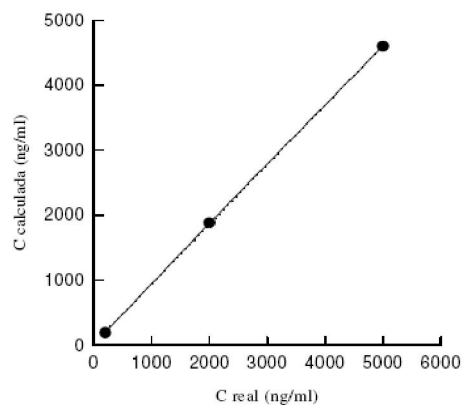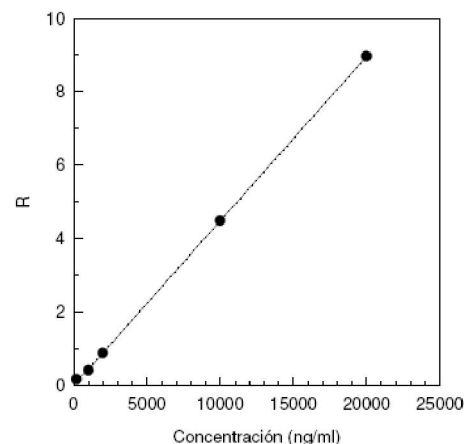
each pattern is indicative of the accuracy of the analytical method. The coefficient of variation, on the other hand, is indicative of the accuracy of the method.

RESULTS AND DISCUSION

The results obtained in the test of accuracy and precision of the analytical method used in the evaluation of busulfan in plasma samples are illustrated in TABLE 1. Each pattern was prepared seven times.

The accuracy and reproducibility of the chromatographic method used for the evaluation of busulfan in plasma samples can be regarded as acceptable, judging by the results shown in TABLE 1.

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The coefficient of variation obtained after preparing seven times the same pattern (indicative of the reproducibility of the analytical method) was between 5 to 8%. Deviations from the average values of experimentally determined concentration (Ccalc.) compared to the theoretical values, (Creal), indicative of the accuracy of the method were between 5 to 8%. The representation of the values of Ccalc against Creal values (Figure 1), and subsequent linear regression allowed to appreciate an excellent correlation between both values, with an intercept and a slope practically zero near the unit.

In Figure 2 are shown busulfan concentrations calculated against the actual for each pattern and the corresponding regression line.

The continuous straight line was obtained by least squares linear regression and its expression is:

$$C_{calc} = 0{,}92 \cdot C_{real} + 21{,}35 \qquad r > 0{,}999$$

The straight line is practically coinciding with the ideal straight, stating:

$$C_{calc} = C_{real}$$

Figure 3 shows, by way of example, the calibration lines obtained for busulfan in plasma as well as the equation that defines it.

The continuous straight line was obtained by least squares linear regression and its expression is:

$$R = 4{,}47 \cdot 10^{-4} \cdot C + 0{,}021$$

The analytical method used in this report for quantification of busulfan in plasma samples (high performance liquid chromatography) has adequate characteristics of accuracy, reproducibility and speed.

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