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High-performance thin-layer chromatography with a derivatization procedure, a suitable method for the identification and the quantitation of busulfan in various pharmaceutical products

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Abstract

Busulfan is an alkylating agent widely used in combination chemotherapy regimens followed by allogeneic or autologous hematopoietic stem cell transplantation (HSCT). We present a rapid method for assaying busulfan in pharmaceutical preparations using high-performance thin-layer chromatography (HPTLC) and derivatization with 4-nitrobenzylpyridine. The method is accurate and precise and allows quantitation of busulfan in aqueous solutions from 100 to 500 μg/ml. It is suitable for identification, quantitation and stability studies of busulfan in pharmaceutical products, i.e. capsules or infusion bags prepared in a hospital pharmacy.

Keywords: Busulfan; HPTLC; Derivatization; 4-Nitrobenzylpyridine; Cyclophosphamide; Quality control

1. Introduction

Busulfan is widely used in combination high-dose chemotherapy regimens followed by allogeneic or autologous hematopoietic stem cell transplantation (HSCT) for the treatment of several diseases, including leukemias [1], solid tumors [2,3] and genetic diseases [4]. For a long time an appropiate busulfan formulation for high-dose chemotherapy (HDC) was lacking. The common dosage for HSCT is 16 doses of 1 mg/kg busulfan, every 6 h over 96 h. Commercially available, 2 mg tablets of busulfan (Myleran®) were developed for conventional chemotherapy but are not practicable for HDC [5]. For instance, a man weighing 70 kg had to swallow 35 tablets of Myleran® per intake. This was considered unacceptable for nursing practices so Hospital Pharmacists developed capsules with a prescribed dose of busulfan in order to solve

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this problem. An intravenous formulation of busulfan, with an orphan drug status, has recently been developed in the United States for the same purpose [6]. In the not too distant future, infusion bags of busulfan will be prepared routinely in Hospital Pharmacies in Europe. An appropriate method is needed to check the dose and the stability of these pharmaceutical preparations. Methods previously developed to quantitate busulfan used gas chromatography (GC) or high-performance liquid chromatography (HPLC). Mass spectrometry (MS), electron capture detection (ECD) or spectrofluorimetry were the methods used for detection purposes. For several of these methods, busulfan had to be chemically derivatized before chromatography either so that it could be measured with a spectrofluorimeter or to make it volatile for GC. These methods are very sensitive and useful for assaying busulfan in biological fluids for pharmacokinetic studies. Calibration curves range from 20 to 2500 ng/ml but this range of concentration is not appropriate for determining the amount of busulfan in pharmaceutical products without considerable prior diluting. Furthermore, most of these methods are time-consuming (for review [7]).

Here, we describe a method that is rapid and suitable for identifying and quantifying busulfan in pharmaceutical preparations, i.e. capsules or infusions bags, manufactured in a Department of Clinical Pharmacy (DCP). The method uses high-performance thin-layer chromatography (HPTLC) before a derivatization procedure using 4-nitrobenzylpyridine [8,9]. The range of concentration, set from 100 to 500 μg/ml, is adequate for pharmaceutical applications.

This work is part of the quality control program for pharmaceutical products prepared in the DCP at the Institut Gustave Roussy [10–12].

2. Materials and methods

2.1. Materials and chemicals

2.1.1. Chemicals and solvents

Busulfan (FLUKA, Steinheim, Ge, purity >99%), cyclophosphamide (ENDOXAN®, Asta Medica, Merignac, Fr), and 4-nitrobenzylpyridine (4-NBP) (Aldrich, purity >99%) were used to prepare both standards and the derivatization mixture. Triethylamine and ethanol were analysis grade (Carlo Erba, Rodano, It). Organic solvents, i.e. methanol, acetonitrile, ethyl acetate, chloroform and isopropanol were HPLC grade (Carlo Erba, Rodano, It). Polyethylene glycol 400 (Sigma, St Louis, USA), *N*,*N*-dimethylacetamide (Merck, Hohenbrunn, Ge), 1,4-butanediol (Aldrich, Steinheim, Ge), tetrahydrofurane (Merck, Darmstadt, Ge), heptane sulfonic acid (Sigma, St Louis, USA), lactose (Cooper, Melun, Fr), carmin (Cooper, Melun, Fr) and dextrose (Macopharma, Fr) were analysis grade.

2.1.2. HPTLC CAMAG® analytical station

HPTLC CAMAG® (Chromacim, Lyon, Fr) is composed of five separated modules: (1) an HPTLC-Vario® chamber for optimization of mobile phases, (2) two TLC sampler III® automated sampler applicators, (3) five solid Teflon (PTFE) migration chambers, (4) a TLC Scanner 3® densitometer controlled by the CATS 4® software (4.05 version) and (5) a TLC plate heater III®.

2.1.3. Stationary phase

The stationary phases (Lichrospher® Si60 F254 nm), manufactured by Merck, were made of uniform thin silica layers that were placed on a 0.2 mm glass surface. The granulometry phase (6 nm) is guaranteed by the manufacturer and its homogeneity is one of the key factors contributing to quality separation.

2.2. Methods

2.2.1. Qualitative and quantitative HPTLC densitometric assay

2.2.1.1. Samples, standards and quality control preparations (QCs). Busulfan (Fig. 1) stock solutions (5 mg/ml) were prepared using crystalline powder (50 mg) dissolved in acetonitrile. Busulfan

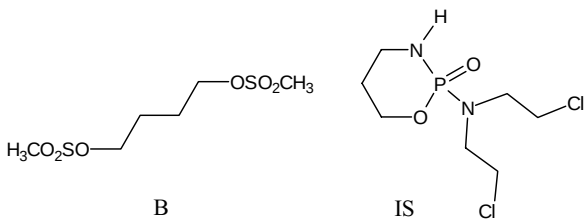

Fig. 1. Chemical structures of busulfan (B) and cyclophosphamide (IS).

standards were made by diluting stock solution in a methanol/water (50:50 (v/v)) mixture. Standard concentrations were set at 100, 200, 300, 400 and 500 μg/ml. Three quality control preparations (QCs), set at 150, 250 and 450 μg/ml were prepared from another stock solution, according to the same procedure. The 10 mg/ml internal standard (IS) stock solution was prepared using commercially available cyclophosphamide (ENDOXAN®, Fig. 1) diluted in injectable water, as specified by the manufacturer. The internal standard working solution was prepared by diluting stock solution in the water/methanol 50/50 mixture to obtain 4 mg/ml. Samples, standards and QCs were spiked with the IS working solution in a constant volume ratio (10:1 (v/v)). All these solutions were transposed in glass snap-ring clipped vials which were arranged on the autosampler's rack.

2.2.1.2. Sample application. HPTLC-Camag system can be used for quantitation purposes because of its accuracy (up to a nanoliter) and the reproducibility of the autosampler. Automated TLC sampler III® devices take into account defined parameters such as the volume, size of the sprayed band and accurate positioning on the chromatography plate. These parameters were computerized by ATS III® software. The system is washed with an isopropanol/methanol/water mixture (33:33:33 (v/v/v)) between each deposition. Two microliters of solution are sprayed onto the plate to form 4 mm bands, 6 mm apart. During the same run, it is possible to assay five standards, two QCs and 20 samples placed within the QCs.

2.2.1.3. Mobile phase and migration. We developed an ethyl acetate/chloroform/methanol mixture (65:20:15 (v/v/v)) for busulfan and cyclophosphamide. We were able to separate the two compounds completely using a 5 min horizontal sandwich migration method.

2.2.1.4. Busulfan and the IS derivatization procedure. After migration, the chromatography plate was dried with a hair drier. Derivatization was performed by automated soaking of the chromatography plate in 4-NBP ethanolic solution (2% (w/v)). The chromatography plate was dried a second time with a hair drier and heated at a temperature of 193 °C for 10 min on a TLC plate heater III®. When the plate had cooled down to room temperature, the derivatized spots were revealed by soaking it in a triethylamine–ethanol solution (50/50 (v/v)) and it was finally dried with a hair drier.

2.2.1.5. Densitometric analysis. The plate was analysed on a TLC Scanner 3® densitometer controlled by the CATS 4® software (4.05 version). The densitometric analysis was performed at 600 nm. This was a compromise so that both derivatized busulfan and cyclophosphamide products would be detected.

2.2.1.6. Identification and quantitation procedure. The surface area of both busulfan and cyclophosphamide were automatically measured for each sample. Busulfan/cyclophosphamide ratios were calculated for each standard, each QC and each sample. The calibration curve was set taking these ratios into account. QCs and samples were calculated according to the response ratio and the calibration curve equation. Each compound is defined according to its retardation factor (R_f) which is the ratio between substance migration and solvent migration distances. With this parameter, busulfan can be qualitatively assayed in pharmaceutical preparations.

2.2.2. Validation of the method

2.2.2.1. Selectivity. We analysed many chemical impurities or related substances which may be present in busulfan pharmaceutical products and other alkylating agents.

2.2.2.2. Calibration and internal standard. The calibration function, i.e. relationship between busulfan/cyclophosphamide peak area ratios and the amount of busulfan applied was determined by linear regression over a defined range from 100 to 500 μg/ml. Each calibration curve was validated using two QCs prepared at 150 and 450 μg/ml. Samples were placed on the rack within the QCs. Six calibration curves were done to ensure that the linear regression model was the most accurate for quantitation purposes.

2.2.2.3. Accuracy. Accuracy provides information about the recovery of the analyte from the sample through the analysis of in-system calibration of sample solutions of known substance content. The solutions were spiked with three different known concentrations

of busulfan, i.e. 150, 250 and 450 μg/ml, respectively. These low, medium and high QCs, were analysed individually six times. Mean and relative standard deviations (R.S.D.) were calculated. In order to confirm these results, and after dilution (1/500), we assayed each standard by gas chromatography coupled to mass spectrometry using a deuterated internal standard [13].

2.2.2.4. Precision. In accordance with International Conference of Harmonisation Guidelines (ICH Q2A and Q2B), precision includes three components: repeatability, intermediate precision and reproducibility. Here, reproducibility was not studied.

2.2.2.5. Repeatability. Repeatability, expressed as the relative standard deviation (R.S.D.) or coefficient of variation of repeatability (CV_r), consists in multiple measurements of a homogenous sample under the same analytical conditions with the same equipment and in the same laboratory. The analysis of each sample was repeated six times.

2.2.2.6. Intermediate precision. Intermediate precision evaluates the reliability of the method in a different environment from that used during the development of the method. Determination, expressed as the R.S.D. or coefficient of variation of intermediate precision (CV_i), consists of six measurements of each level studied, i.e. QCs under the same analytical conditions but on multiple days, by different analysts and different equipment except for the HPTLC workstation.

3. Results and discussion

3.1. Selectivity and specificity

Busulfan and cyclophosphamide (IS) were separated by baseline resolution, as shown in Fig. 2. Cyclophosphamide and busulfan retardation factors were, respectively, 0.45 and 0.6. We also verified that there was no interference with dextrose, 1,4-butanediol, tetrahydrofurane, lactose, carmin, polyethylene glycol 400, *N*,*N*-dimethylacetamide and sulfonic acids [14]. These compounds are impurities or chemicals that may be found in pharmaceutical preparations of busulfan.

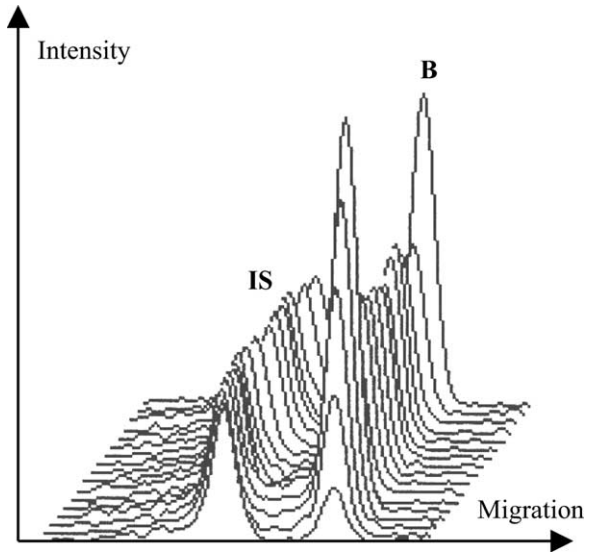

Fig. 2. Superposition of 17 chromatograms with busulfan and IS by HPTLC after both derivatization and revelation procedures. This figure presents the superposition of 17 chromatograms with cyclophosphamide (IS) and busulfan (B). The densitometric analysis was performed at 600 nm on a TLC Scanner 3® densitometer. The first six chromatograms were the standards (5) and the first QC set at 150 μg/ml. The following chromatograms (10) were corresponding to 10 busulfan capsules. The sequence was finished by a second QC set at 450 μg/ml.

3.2. Validation of busulfan quantitation

3.2.1. Calibration

The calibration function was determined by linear regression over 100–500 μg/ml with a $r^2 \geq 0.99$. The coefficient of variation (CV) of peak area ratios was calculated for each standard level (Table 1). All CV were very close to 5% for standards between 200 and 500 μg/ml. Nevertheless, the CV for the 100 μg/ml standard was 15.6% and, thus, to be the must accurate,

Table 1
Variation of standard peak area ratios

Concentration (μg/ml)	R.S.D.	CV (%)
100	5.7	15.6
200	4.1	5.5
300	5.6	4.5
400	7.8	4.6
500	14.0	6.5

Values calculated were based on six different measurements.

Table 2
Results of the accuracy study

QC (μg/ml)	Mean	R.S.D.	Bias (%)
150	150.6	5.8	+3.9
250	254.2	9.7	+3.9
450	476.9	14.5	+3.2

Values calculated were based on six different measurements.

it is convenient to define for the routine use a target amount in the middle range of the calibration curve.

3.2.2. Accuracy

The results are summarized in Table 2. Means and R.S.D. values were calculated from the six determinations of each QC (150, 250 and 450 μg/ml). The method was considered accurate according to the values which were ≤4%. The accuracy of the method was confirmed by a method previously validated with GC–MS using a deuterated internal standard (busulfan-d4). For the GC–MS analysis, HPTLC standards were diluted 500-fold to obtain a concentration within the range of the GC–MS calibration curve, i.e. from 50 to 2000 ng/ml. We plotted the curve corresponding to GC–MS results (Y-axis) versus HPTLC standard values (X-axis) and excellent linearity ($R^2 = 0.9995$) was demonstrated with a slope of 0.9987 which is very close to the target value of 1 (equation: $Y = 0.9987X - 8.1032$). This demonstrates that our HPTLC method for pharmaceutical applications was accurate compared to the standard method used to determine busulfan pharmacokinetics in our institution.

Table 3
Results of the repeatability (CV_r) and the intermediate precision (CV_i) studies

QC (μg/ml)	CV_r (%)	CV_i (%)
150	4.3	3.8
250	4.6	3.8
450	4.3	3.0

Values calculated were based on six different measurements.

3.2.3. Precision

The CV values for repeatability (CV_r) and for intermediate precision (CV_i) are summarized in Table 3. The method can be considered precise since all of these values were below 5%.

3.3. Application: examples

3.3.1. Quality control of busulfan capsules

In our institution, oral capsules with a prescribed dose of busulfan are frequently prepared for patients undergoing HDC. To guarantee the quality of the preparation, we verified mass uniformity of 20 randomized capsules in all batches, as specified by the European Pharmacopeia (IIIrd edition). To complete the validation procedure, we checked the amount of busulfan in 10 randomly selected capsules among the prepared capsules in some batches as specified by the French Pharmacopeia (Xème édition). The results of this test are presented in Table 4. The calculated amount were easily included in the acceptance criteria defined by the French Pharmacopeia, i.e. no more than one capsule outside the 85–115% limit

Table 4
Dose in 10 busulfan capsules with 70 mg as the theoretical value

Capsules	Busulfan (mg)	Amount (μg/ml)	Target amount[a] (μg/ml)	Calculated amount (μg/ml)	Δ (%)
1	70	1400	280	264	−6
2	70	1400	280	272	−3
3	70	1400	280	269	−4
4	70	1400	280	273	−2
5	70	1400	280	275	−2
6	70	1400	280	289	3
7	70	1400	280	271	−3
8	70	1400	280	276	−1
9	70	1400	280	276	−1
10	70	1400	280	281	0

The powder content in each capsule is poured into 50 ml of acetonitrile.
[a] This busulfan solution is thus diluted in a water–methanol mixture (1/5) to reach a target amount.

but within the 75–125% limit of the target value. In fact, the Δ (%) between the calculated value and the theorical value were always below 7%. To assay busulfan content, capsules were opened and the powder, i.e. busulfan and lactose, was poured into 50 ml of acetonitrile. Busulfan dissolved in acetonitrile and lactose remained suspended. This acetonitrile preparation was correctly diluted in the water–methanol mixture to obtain a concentration between 100 and 500 μg/ml. With this diluting step, the suspended lactose dissolved in the water–methanol mixture. The sample was then assayed by our HPTLC method.

3.3.2. Checking infusion bags

We demonstrated that there was no interference with polyethylene glycol 400 and *N*,*N*-dimethylacetamide. These solvents are mixed with dextrose 5% or sodium chloride 0.9% in a new intravenous busulfan formulation (Busilvex®). Infusion bags contain busulfan at a quasi-constant concentration of 0.55 g/l and content determination is made after a 1/2 diluting step in water–methanol mixture. Our procedure will soon be useful to verify busulfan infusion bags manufactured in the DCP of Institut Gustave Roussy.

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