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Journal of Chromatography B, 775 (2002) 189–195

JOURNAL OF CHROMATOGRAPHY B

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Validated liquid chromatographic method for the determination of bexarotene in human plasma

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Received 25 February 2002; received in revised form 24 April 2002; accepted 3 May 2002

Abstract

A new liquid chromatographic method is described for the determination of the anti-tumour agent bexarotene in human plasma over the range 0.500–1500 ng/ml, using 1 ml of sample. Sample preparation consists of liberating the analyte from plasma lipids by adding acetonitrile, followed by acidification of the plasma and liquid extraction using a mixture of isoamyl alcohol and pentane or hexane. Separation and quantitation are performed by reversed-phase column liquid chromatography with fluorescence detection. Parameters affecting the performance of these steps are discussed. Validation results on linearity, selectivity, accuracy, precision, recovery and stability are shown, as well as the application of the method to samples from clinical trials.

Keywords: Bexarotene

1. Introduction

Bexarotene (Fig. 1) is a new synthetic retinoid analogue. It was found to have anti-tumour activity, established via a novel mechanism of action [1], in combination with a favourable toxicity profile and is now being investigated as a potential therapy for oncologic, dermatologic and metabolic indications. Bexarotene is the active ingredient in Targretin capsules, which have been approved in the US and European community for the treatment of select patients with cutaneous T-cell lymphoma (CTCL), and in Targretin gel, which has been approved in the US for the same indication. The recommended oral dose of bexarotene is 300 mg/m^2 once daily which can result in peak plasma concentrations of up to 1500 ng/ml and trough levels in the order of 5 ng/ml. Following topical application much lower plasma concentrations are generally observed and sub-ng/ml trough levels are frequently found [2].

For the determination of pharmacokinetic parameters after administration of bexarotene, a gas chromatography–mass spectrometry (GC–MS) method has been described, which allows the determination of bexarotene in human plasma down to 1 ng/ml [3]. Briefly, this method is based on extraction of the analyte from acidified plasma by 1-chlorobutane, followed by evaporation of the extractant, derivatization of bexarotene to its methyl ester, reconstitution in chloroform and injection into the GC–MS system. Although the sensitivity of this method is adequate for the determination of relevant plasma levels after

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00291-X

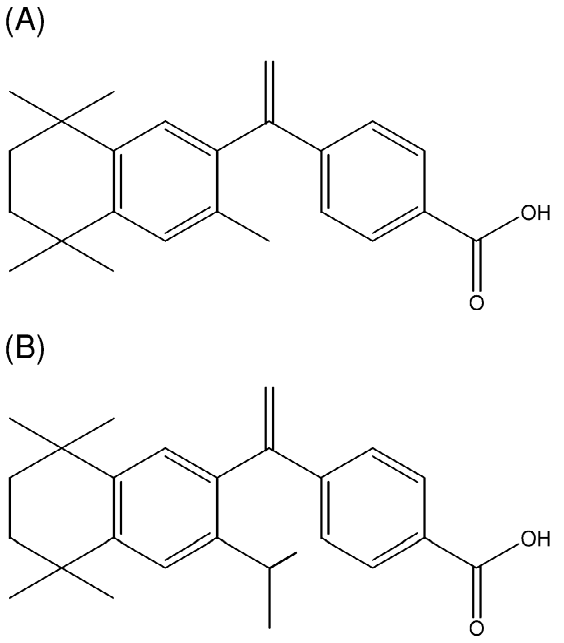

Fig. 1. Structure of bexarotene (A) and LG100130, the internal standard (B).

oral administration, additional sensitivity is desired to support studies with topical application. In addition, the derivatization step makes it a lengthy and cumbersome procedure.

In order to avoid the derivatization step and simplify the analytical methodology, the use of a liquid chromatographic (LC) procedure could be an option. For the determination of bexarotene in (0.5 ml) dog plasma samples, a straightforward method has been described [4], which uses protein precipitation with acetonitrile for sample preparation. The supernatant is evaporated and reconstituted in mobile phase prior to injection into an LC system with UV detection at 262 nm. The sensitivity of this approach, however, is far from sufficient for human plasma, since the quantitation limit is as high as 216 ng/ml. In order to be able to quantify bexarotene in human plasma at the relevant levels, it is obvious that this method should be considerably adapted, with regard to both the sample preparation procedure and the detection mode.

In this paper, a method is described for the determination of bexarotene in human plasma. For the support of clinical studies with oral or topical administration, two appropriate calibration ranges are used: a high-concentration range and a low-concentration range, respectively. Special attention is paid to the sample preparation procedure, which turned out to be especially important. To illustrate the applicability of the method, the validation results are presented and the use of the method for clinical study samples is shown.

2. Experimental

2.1. Chemicals

Bexarotene and LG100130 (the internal standard, Fig. 1) were obtained from Ligand Pharmaceuticals (San Diego, CA, USA). Acetonitrile, hydrochloric acid and acetic acid were purchased from J.T. Baker (Deventer, The Netherlands) and *n*-pentane from Lab Scan (Dublin, Ireland). Ammonium acetate, *n*-hexane and isoamyl alcohol came from Merck (Darmstadt, Germany). Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Standard solutions, calibration and validation samples

A methanolic stock solution (containing 500 mg/l of bexarotene) was diluted with methanol to 2.5 mg/l. Both solutions were used to prepare calibration and validation samples by adding small volumes to blank heparinized human plasma. Calibration samples with the following concentrations were used for the high-range method: 3.00, 10.0, 25.0, 100, 400, 800, 1200 and 1500 ng/ml. For the low-range method, the concentrations were: 0.500, 1.50, 4.00, 10.0, 25.0, 50.0, 100 and 150 ng/ml. In addition, validation samples, prepared from a separate weighing, were used at concentrations of 3.00, 10.0, 400 and 1200 ng/ml (high-range method) or 0.500, 2.00, 60.0 and 120 ng/ml (low-range method).

A stock solution of the internal standard, LG100130, (50 mg/l) was prepared in methanol and (for the low-range method) used to prepare a working solution of 2.5 mg/l.

2.3. Equipment

A Waters (Milford, MA, USA) Model 717 autosampler was used to introduce 50 μl of the pretreated samples into the chromatographic system. Reversed-phase LC was performed using a 5 μm Inertsil ODS-2 column (150×4.6 mm I.D.), obtained from Chrompack (Middelburg, The Netherlands), which was conditioned at 35 °C by a Julabo (Seelbach, Germany) waterbath. A Waters Model M510 pump was used to deliver the eluent, a mixture of 10 m*M* ammonium acetate, acetonitrile and acetic acid (1:4:0.04, v/v). For the high-range method, detection was performed with a Jasco (Hachioji, Japan) Model 831 FP fluorescence detector, for the low-range method with a (more sensitive) Waters Model 474 fluorescence detector. In both cases, the excitation wavelength was 260 nm and the emission wavelength 430 nm.

2.4. Sample preparation

An aliquot of 1000 μl plasma was mixed with 20 μl internal standard solution. After vortex-mixing for 10 s, five aliquots of 250 μl acetonitrile and one aliquot of 1000 μl 0.5 *M* hydrochloric acid were added, each followed by short vortex-mixing. Subsequently, 5 ml extraction solvent (a mixture of *n*-pentane and isoamyl alcohol, 98:2, v/v, for the high range and a mixture of *n*-hexane and isoamyl alcohol, 98:2, v/v, for the low range) was added and extraction was performed for 20 min in a rotating tube rack at 45 rev./min. After centrifugation for 10 min at 3200 *g*, the aqueous layer was frozen and the organic phase transferred to a new tube and evaporated to dryness under nitrogen at 40 °C. The residue was redissolved in 400 μl acetonitrile by vortex-mixing for 30 s and ultrasonication for 2 min, 100 μl of 10 m*M* ammonium acetate solution were added and the mixture was centrifuged for 5 min at 13,000 *g*. An aliquot of 50 μl was injected onto the LC system.

2.5. Validation experiments

The low-range method was validated over the range 0.500–150 ng/ml and the high-range method over the range 3.00–1500 ng/ml, according to the validation approach as described by Wieling et al. [5]. The peak height ratio of bexarotene over internal standard was plotted as a function of its concentration, using eight calibration samples in triplicate and a weighting factor of $1/x$ for the low-range method and a weighting factor of $1/x^2$ for the high-range method. The linearity of the method was established from these data by performing a goodness of fit and lack of fit test by analysis of variance (ANOVA). The selectivity of the method was checked by analysing drug-free human plasma samples from six different healthy volunteers. Accuracy and within-run, between-run and overall precision were calculated by ANOVA at four validation levels (0.500, 2.00, 60.0 and 120 ng/ml for the low range and 3.00, 10.0, 400 and 1200 ng/ml for the high range), by analysing samples in triplicate during five analytical runs performed on 5 different days. The recovery was determined at 10.0, 400 and 1200 ng/ml (high range) and at 2.00 ng/ml (low range) by comparing the response of the validation samples to the response of injections of standards at the same level performed in the same run. The stability of bexarotene was assessed at three concentration levels (2.00 ng/ml using the low-range method and 10.0 and 1200 ng/ml using the high-range method). Freeze–thaw stability in plasma was determined by repeatedly ($n=5$) freezing and thawing plasma samples and analysing the samples in triplicate after each freeze–thaw cycle. The stability of bexarotene in processed plasma at 10 °C, during storage in the autosampler, was performed by repeated injection of pooled extracts every 2 h for a period of 30 h. The stability of bexarotene in plasma at ambient temperature was assessed by processing and analysing plasma samples in triplicate after storage for 24 h on the laboratory bench.

3. Results and discussion

3.1. Sample preparation

Considering the relatively low levels at which bexarotene has to be determined, it was considered important to develop a method with a high analyte recovery. Since bexarotene is an acidic ($pK_a=5.54$) and highly lipophilic (log $P=4.34$) compound,

liquid extraction with a relatively non-polar extractant from acidified plasma seemed a logical first choice for sample preparation. Therefore, the sample preparation procedure described for the GC–MS method, extraction with 1-chlorobutane after acidification with 0.5 *M* hydrochloric acid [3], was initially tried. In this way, bexarotene could be essentially completely extracted from aqueous samples, but from spiked plasma the recovery was only 15% (Fig. 2A). The use of a less polar extractant, hexane, did not improve the recovery and it was therefore concluded that bexarotene most probably binds to plasma constituents and that its binding is not disrupted by the addition of an extraction solvent.

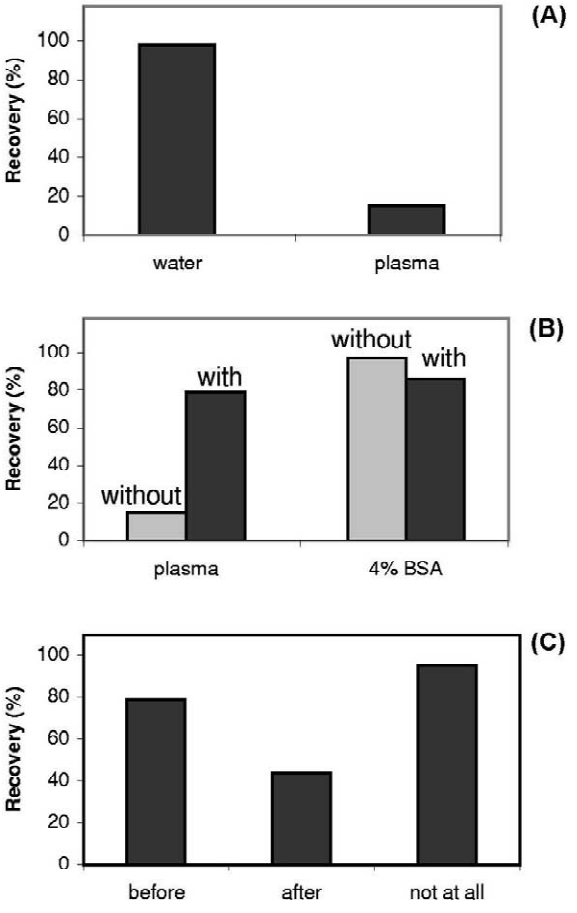

Fig. 2. (A) Analyte recovery from water and from untreated plasma; matrix effect on the extraction with chlorobutane following acidification; (B) analyte recovery from plasma and from an aqueous 4% BSA solution; the effect of the addition of acetonitrile prior to acidification and extraction with hexane; (C) analyte recovery from plasma, the effect of protein removal after addition of acetonitrile on the extraction with hexane; protein removal before or after acidification or not at all.

In order to liberate bexarotene from its possible binding site on plasma proteins and thus improve the extractable fraction, protein precipitation was performed prior to liquid extraction with hexane. Deproteinization with perchloric acid, which has the advantage of both removing the proteins and acidifying the remaining supernatant, was attempted first, but yielded a very low recovery of only 5%. Deproteinization with acetonitrile, however, followed by removal of the protein precipitate, acidification of the supernatant and extraction with hexane resulted in recoveries of typically 80% (Fig. 2B). Apparently, bexarotene cannot be released from its binding site by just denaturating the plasma proteins, which implies that the observed tight binding is not to a common protein such as albumin. This was confirmed by spiking bexarotene to an aqueous 4% bovine serum albumin (BSA) solution. The recovery obtained by just extracting the sample with hexane was very good (95%) and this was not improved by first precipitating the protein with acetonitrile (85%) (Fig. 2B).

Further, it was observed that a much lower recovery (typically 30%) was obtained when bexarotene was spiked to plasma samples with a high lipid content (from patients with advanced cancer) than when it was spiked to plasma from healthy volunteers (typically 80%). This indicates that the compound most probably binds to plasma lipids or lipoproteins or that the presence of lipids affects the extraction efficiency of bexarotene from the matrix. This would also explain why the addition of acetonitrile helps to improve the recovery: the organic modifier probably breaks up the plasma lipid structure and releases bexarotene.

A final important observation is related to the acidification of plasma. When the protein precipitate, formed after acetonitrile addition, was removed by centrifugation and the remaining supernatant acidified and extracted, a recovery of about 80% was found. However, when the plasma sample was acidified directly after the addition of acetonitrile and the protein precipitate was then removed, the recovery was found to drop to ~40%. When the protein precipitate was not removed at all, i.e. when

the extraction solvent was added to plasma immediately after the addition of acetonitrile and acid and the extraction performed in the presence of the denaturated protein fraction, the recovery was typically 90% (Fig. 2C). From these findings it can be concluded that bexarotene interacts with the protein precipitate in its neutral form (at low pH) but not in its negatively charged form (at physiological pH). When the protein precipitate is removed at a low pH, a large part of the (neutral) analyte molecules are thus lost along with the precipitate, whereas at a higher pH most of the (charged) analyte molecules remain in the sample. The best option, however, seems to be to leave the protein precipitate in the tube when performing the extraction.

The above results indicate that the order in which the different sample preparation steps are performed is crucial for a high recovery and, consequently, for a sensitive assay. The final procedure consisted of the following steps: addition of acetonitrile (1250 μl) to a 1000-μl plasma sample, immediately followed by the addition of 1000 μl 0.5 *M* hydrochloric acid and 5 ml extraction solvent. The use of a mixture of pentane and isoamyl alcohol (98:2, v/v) turned out to give good results and clean chromatograms and was used for the validation of the high range method. When for the validation of the low range method another lot of pentane was used, this gave rise to interfering peaks in the chromatograms and a mixture of hexane and isoamyl alcohol was used instead. The assay performance was found to be comparable, which illustrates the robustness of the sample preparation procedure.

3.2. Chromatography and detection

The chromatography and detection of bexarotene turned out to be rather simple to optimize. With a standard 5 μm octadecyl column (150×4.6 mm I.D.) and using an acidic mobile phase with a high modifier content (80% acetonitrile) the non-polar analyte could be very well resolved from endogenous plasma components and the internal standard. The analytical run time of 11 min was considered acceptable for the present application; in actual practice, a typical sample throughput of 80 samples per day is routinely achieved. The maximum UV absorption wavelength of 262 nm, which was used for the determination of bexarotene in dog plasma [4], was not sufficient for the quantification at the relevant levels for clinical studies in humans. However, an approximate 10-fold increase in signal-to-noise ratio could be obtained when fluorescence detection was used. The optimum excitation and emission wavelengths were found to be 260 nm and 430 nm, respectively. An additional advantage with fluorescence detection was the gain in selectivity: the detection response of endogenous plasma components was significantly reduced and very clean chromatograms were generally obtained. To illustrate this, Fig. 3 shows chromatograms of blank plasma, plasma spiked at 3.00 ng/ml and a plasma sample taken from a subject after administration of 75 mg bexarotene. The peaks eluting in the beginning of the chromatogram for study samples are most probably hydroxylated and oxo-metabolites of bexarotene,

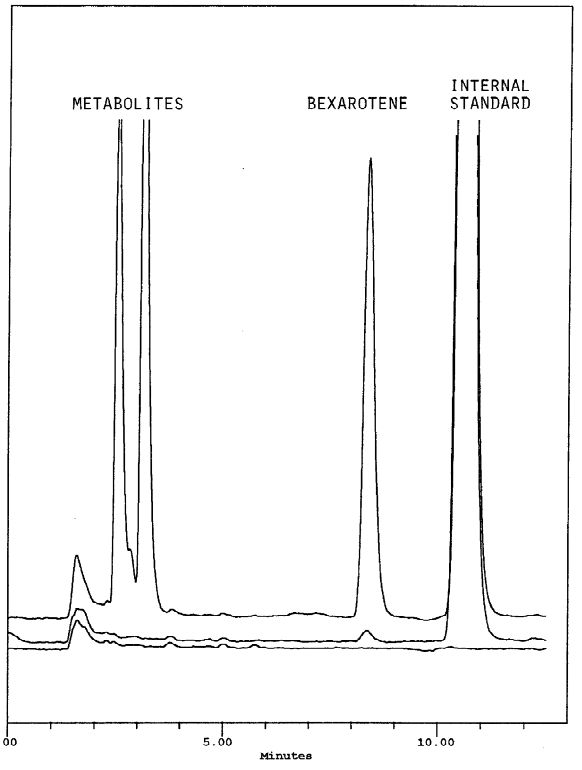

Fig. 3. LC-fluorescence chromatograms of blank plasma (lower trace), plasma spiked with bexarotene at 3.00 ng/ml (middle trace) and plasma taken from a healthy volunteer after oral administration of bexarotene (upper trace).

since chromatograms of standard solutions of these compounds were found to contain peaks at the same retention times.

3.3. Validation results

The linearity of the method was established over two concentration ranges: 0.500–150 ng/ml and 3.00–1500 ng/ml, for the support of studies with topical and oral administration, respectively. Analysis of variance indicated that the linear model with a weighting of $1/x$ for the low range and $1/x^2$ for the more extended high range was appropriate for establishing a relationship between concentration and detector response. The goodness of fit was highly significant and correlation coefficients above 0.998 were found for both ranges.

The selectivity of the method was good, no interfering compounds were found in the plasma samples of different healthy subjects at the retention time of either bexarotene or the internal standard. The detection limit of the low range at S/N 3 was estimated to be 0.3 ng/ml. The lowest point of the calibration curve (0.500 ng/ml) was taken as the lower limit of quantitation and the accuracy and precision at this level were well within the limits (bias and C.V.$<$20%) established for this purpose [6]. Precision and accuracy were also satisfactory at the other levels studied. For both ranges, the values were typically better than 10% (see Table 1).

The recovery was found to be high and consistent over the entire concentration range studied (2.00–1200 ng/ml), with an average of 92.3% and a C.V. of 3.6%. When processed samples were stored at 10 °C in the autosampler, bexarotene showed a very good stability: at the concentrations studied (2.00, 10.0 and 1200 ng/ml) the responses varied less than 3% within 30 h of storage. After storage at ambient temperature for 24 h, bexarotene concentrations in plasma deviated by no more than 1.0% from the concentrations in unstored plasma. In addition, no major effect of repeated freezing and thawing was observed. At 10.0 and 1200 ng/ml, a bias of not more than 4% from the nominal value was found for five freeze–thaw cycles. At 2.00 ng/ml, a deviation of up to 10% was found for four cycles, with a deviation of −22.5% for the fifth cycle, which probably can be regarded as an outlier.

Table 1
Summary of precision and accuracy for bexarotene

Nominal concentrations (ng ml^{-1})	Concentrations			
	Bias (%)	Within-run precision (%)	Between-run precision (%)	n
High concentration range				
3.00	−1.2	7.4	6.8	15
10.0	−5.7	2.0	5.0	15
400	+2.0	0.6	0.8	15
1200	+1.3	1.1	0.8	15
Low concentration range				
0.500	−1.9	8.8	12.8	15
2.00	+6.3	7.4	8.2	15
60.0	+1.3	8.9	10.1	15
120	+5.9	1.3	6.0	15

3.4. Application to clinical samples

To date, approximately 2000 samples from clinical trials with bexarotene have been analysed using the described methodology. For all analytical runs, calibration curves were recorded with coefficients of correlation above 0.997. Per analytical run, quality control samples were analysed in duplicate at three levels, which were similar to the three highest validation levels. The average accuracy (expressed as percentage bias from the nominal value) and precision (expressed as percentage C.V.) found for the quality control samples were −2.9% and 5.7% (10.0 ng/ml), +0.8% and 3.4% (400 ng/ml), +0.2% and 3.1% (1200 ng/ml), +17.7% and 5.2% (2.00 ng/ml), +6.6% and 1.6% (60.0 ng/ml) and +8.9% and 3.9% (120 ng/ml), with $n=90$ for the high range levels and $n=10$ for the low range levels.

As an illustration of the applicability of the method, Fig. 4 shows a typical plasma concentration vs. time profile for bexarotene after an oral dose of 75 mg bexarotene. It was administered as a soft gelatin capsule to a healthy volunteer, who gave institutionally approved written informed consent prior to admission to the study.

4. Conclusions

A straightforward and robust LC method has been developed and validated for the determination of

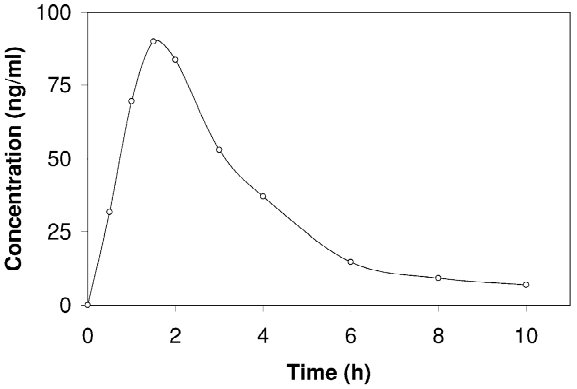

Fig. 4. Plasma concentration–time profile for bexarotene, obtained after a single oral dose of 75 mg in the form of a soft gelatin capsule (○).

bexarotene in human plasma. It allows the quantitation of bexarotene down to 0.500 ng/ml, which is adequate for the support of clinical studies with both oral administration and topical application of bexarotene.

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