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High-performance liquid chromatography analysis of Bobel-24 in biological samples for pharmacokinetic, metabolic and tissue distribution studies

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Abstract

A rapid and simple HPLC method is described for the determination of Bobel-24 (2,4,6-triiodophenol) and other iodinated derivatives in biological samples. The sample preparation was liquid–liquid extraction before injection onto the HPLC system. 2,6-Diiodo-4-methylphenol was used as internal standard. Separation was obtained using a reversed-phase column under isocratic conditions. The mobile phase consisted of water–acetonitrile (62:38). 2,4,6-Triiodophenol was detected at 277 nm. This method was used for Bobel-24 determination in plasma, urine, synovial liquid and different tissues. The assay was applied to pharmacokinetic studies in dog and horse plasma and different dog tissues for tissue distribution profiles toxicological and metabolic studies. In addition, this method for biological samples can be applied to non-biological samples such as pharmaceutical formulations in stability studies and quality control assays. © 1998 Elsevier Science B.V.

Keywords: Bobel-24; 2,4,6-Triiodophenol

1. Introduction

Bobel-24 is a new drug whose antiinflammatory properties have been tested ''in vivo'' in different animal inflammation models. Some interesting properties have recently been described for phenolic compounds related to the structure shown in Fig. 1. It has been found that Bobel-24 is a 5-lipoxygenase inhibitor [1] which could be effective in the therapy of inflammatory diseases mediated by leukotrienes. Presently, studies are being carried out to demonstrate whether Bobel-24 is effective in the treatment

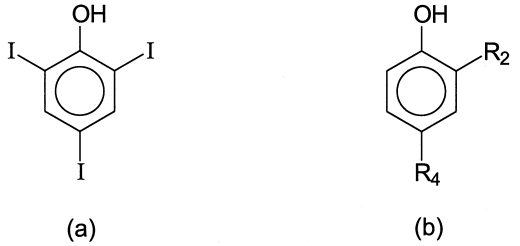

Fig. 1. (a) Structure of Bobel-24. (b) Structure of 2,4-disubstitutedphenol (R_2=alkyl group or halogen; R_4=alkyl group or halogen).

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of articular diseases and seborrhoeic dermatitis in dogs.

Such studies require a simple and rapid analytical method which gives the quantitative determination of Bobel-24 in different biological samples. Peraire et al. [2] described a method using high-performance liquid chromatography (HPLC) with UV absorbance detection for pharmacokinetic studies of Bobel-24 in rat. In the present study we have developed a specific, accurate and reproducible HPLC method which can be applied in both biological and non-biological matrices for the determination of Bobel-24 concentrations. This method has been successfully employed for the analysis of various biological samples obtained from preclinical pharmacokinetic studies, metabolic studies and tissue distribution profiles. It has also been applied in stability studies in pharmaceutical formulations.

2. Experimental

2.1. Reagents and materials

2,4,6-Triiodophenol and 2,6-diiodo-4-methylphenol (the internal standard, I.S.) were synthesized in our laboratory according to a previously described method [1]. Identity of these compound was established by means of their spectral data (IR, ^{1}H NMR, ^{13}C NMR) and purity was checked by thin-layer chromatography (TLC). Phenol was provided by Merck (Darmstadt, Germany). 2-Monoiodophenol was provided by Lancaster (Morecamba, UK). HPLC-grade water and HPLC-grade ethyl acetate were supplied by Promochem (Wesel, Germany). HPLC-grade acetonitrile was provided by Carlo Erba (Milan, Italy). Citrate buffer was prepared with 400 ml of 0.1 *M* citric acid solution and 200 ml of 0.2 *M* dihydrogenophosphate solution. Citric acid and dihydrogenophosphate were supplied by Merck. 0.45-μm membrane filters were provided by Millipore (Cork, Ireland). Vacutainer tubes were provided by Becton Dickinson Vacutainer Systems Europe (Meylan, France).

2.2. Apparatus

The HPLC system consisted of a Waters instrument equipped with computer system for acquisition and integration of data (Maxima 820 chromatography data station), a 510 pump, a 717 injector autosampler, a 486 UV absorbance detector and a Nova Pak C_{18} reversed-phase (4 μm, 150×3.9 mm) column. The homogenizer used for preparations of tissue samples was a Probel with a Heidolph agitator

2.3. Chromatographic conditions

The mobile phase consisted of acetonitrile–water (62:38). The solution was filtered and degassed by vacuum filtering through a 0.45-μm membrane filter. Pump flow-rate was 1.0 ml/min. Peaks of 2,4,6-triiodophenol were detected by UV absorbance at 277 nm. The injection volume was 10 μl.

2.4. Drug standards

Working stock solutions of 2,4,6-triiodophenol and 2,6-diiodo-4-methylphenol were prepared in acetonitrile at a concentration of 2 mg/ml. In order to test the suitability of the system, a chromatographic control was prepared by dilution of the stock solutions with acetonitrile to a final concentration of 5 μg/ml for each compound. Standards of plasma, urine, synovial liquid and different tissues were prepared from the stock solutions using the corresponding drug-free biological fluid to obtain the appropriate final concentrations.

2.5. Biological samples

Blood samples were obtained by extraction (3 ml) and then collected in heparinized vacutainer tubes. Plasma samples were obtained by centrifugation from blood samples. Synovial liquid samples (3 ml) from horses were obtained by direct extraction. Dog tissue samples (kidney, liver, muscle, subcutaneous and heart) were obtained by homogenization with appropriate quantities of citrate buffer (pH 4.5). After obtention all samples were stored at −40°C until analysis.

2.6. Analytical procedure

The I.S. (100 μl of a 10 μg/ml acetonitrile solution) was added to 100 μl of plasma, urine or homogenized tissue sample. After addition of 1 ml ethyl acetate, contents were vortexed for 3 min and

centrifuged at 1000 *g* for 10 min. The organic extracts were removed and dried under nitrogen. Residues were then reconstituted in 50 μl of mobile phase. The injection volume was 10 μl.

2.7. Method validation

The method was validated in plasma for dogs and plasma, urine and synovial liquid for horses [3–6]. Standards of dog plasma (0.5, 1, 5, 10 and 20 μg/ml), horse plasma (0.625, 1.25, 2.5, 5, 10 and 20 μg/ml) and urine (0.1, 0.5, 1, 5, 10 and 20 μg/ml) were prepared and were analyzed, in sextuplicate or triplicate, on three separate days during method validation. Standards of synovial liquid (0.5, 1, 2.5, 5 and 10 μg/ml) were prepared and analyzed in triplicate on two separate days. Linearity of standard curves, intra- and inter-assay precision and accuracy were determined from these data. The limit of quantitation (LOQ) of 2,4,6-triiodophenol was determined from the peak and the standard deviation (S.D.) of the noise level, S_N. The LOQ was defined as sample concentration of 2,4,6-triiodophenol resulting in a peak height of 10-times the S_N. Recovery was determined by comparing the peak heights from processed plasma, urine or tissue standard samples to those from 2,4,6-triiodophenol standards in acetonitrile solution for each standard concentration.

2.8. Quality control and stability

Quality controls (QCs) were prepared at low, medium and high levels for plasma and urine. QCs of synovial liquid were prepared at medium concentration. QC samples were stored at −40°C and analyzed together with corresponding biological samples each day of analysis. The stability of Bobel-24 in biological samples was studied from QC samples [4].

3. Results

3.1. Chromatography

Under the chromatographic conditions described above, the chromatographic control ($n=120$) showed that Bobel-24 was eluted at 5.70±0.13 min and the I.S. at 4.03±0.06 min. Typical chromatograms of plasma and liver from dog are shown in Fig. 2 (chromatograms of blank plasma samples, plasma samples spiked with Bobel-24 and liver samples obtained after intravenous or oral administration). Chromatograms of other dog tissue samples (heart, kidney, muscle, heart and subcutaneous) were similar to those of liver samples. In addition, chromatograms of Bobel-24 in plasma, urine and synovial liquid samples obtained from horses were similar to those obtained in dogs.

3.2. Sensitivity and specificity

The LOQs of Bobel-24 were 0.06, 0.1 and 0.1 μg/ml for plasma, urine and synovial liquid samples, respectively. Specificity of the method was checked. No other interfering peaks were observed in drug-free biological samples. Other derivative products of Bobel-24 (2,6-diiodophenol, 2-monoiodophenol and phenol) were processed under the same chromatographic conditions. These products were eluted at different retention times with a good resolution. Fig. 3 illustrates a chromatogram of acetonitrile solution for three derivative products and Bobel-24.

In the search for degradation products, solutions of Bobel-24 were submitted to stress factors. Chromatograms corresponding to analysis of samples demonstrated the appearance of a peak with a retention time of 2.8 min. The product corresponding to this peak was separated by column chromatography and identified by spectral methods as 2,6-diiodophenol.

3.3. Linearity, precision and accuracy

The assay method has a linearity up to 20 μg/ml for Bobel-24 in biological samples. Results of linearity are shown in Table 1. The precision expressed as the coefficient of variation (C.V.) was calculated for both inter- and intra-assay for each standard concentration. Accuracy was calculated as a relative error for each standard concentration. Results of precision and accuracy for dog plasma and horse plasma, urine and synovial liquid are shown in Table 2.

3.4. Recovery

The extraction efficiencies of Bobel-24 from plasma, urine and synovial liquid were determined for

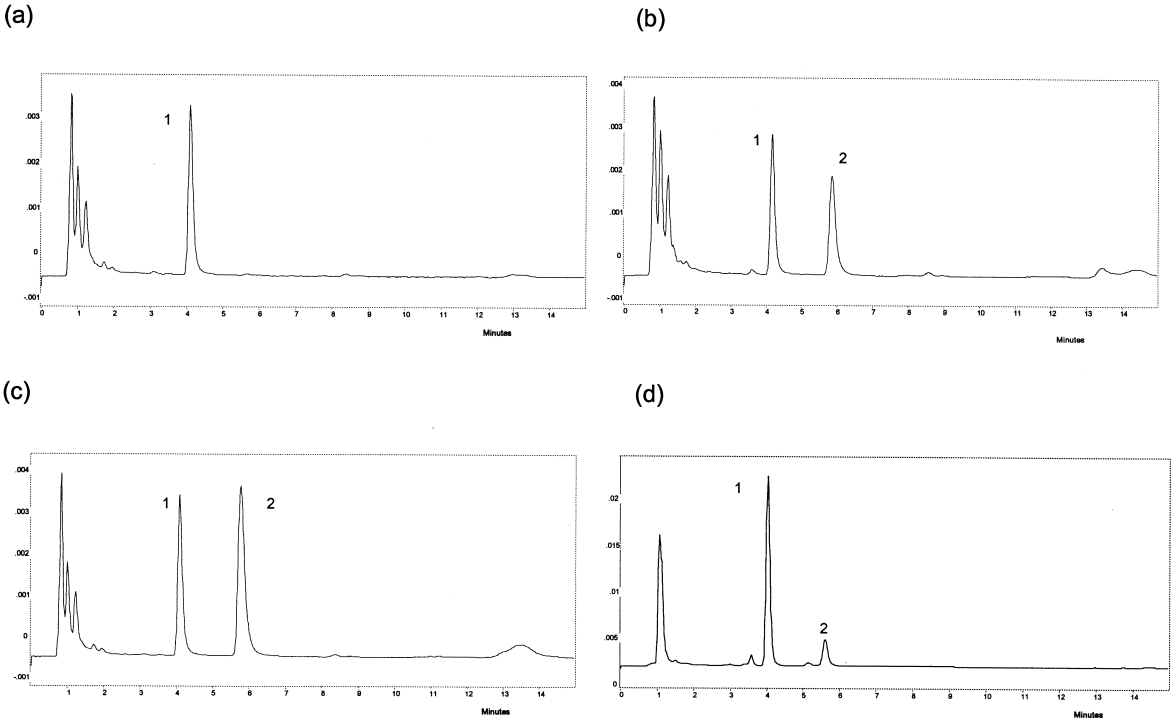

Fig. 2. (a) Chromatogram of blank dog plasma spiked with internal standard 10 μg/ml ml (peak 1=internal standard). (b) Chromatogram of dog plasma spiked with Bobel-24 concentration 10 μg/ml and internal standard 10 μg/ml (peak 1=internal standard; peak 2=Bobel-24). (c) Chromatogram of dog plasma sample 5 min after intravenous administration for pharmacokinetic study (peak 1=internal standard; peak 2=Bobel-24). (d) Chromatogram of dog liver sample 24 h after oral administration for toxicological study (peak 1=internal standard; peak 2=Bobel-24).

each standard concentration from validation standards. No differences were observed between recoveries found for different concentration. The recovery means were 99.8, 90.4 and 90.7% for horse plasma, urine and synovial liquid, respectively.

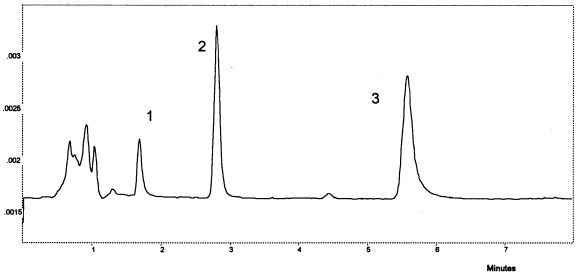

Fig. 3. Chromatogram of three iodinate phenolic derivatives in acetonitrile solution (peak 1=2-monoiodophenol; peak 2=2,6-diiodophenol; peak 3=Bobel-24).

3.5. Quality control and stability

The stability was studied from QC samples over time. Plasma and synovial liquid were stable for at least three months. QC urine demonstrated that urine samples were non-stable under the described conditions. Results of QC plasma and synovial liquid are shown in Table 3.

3.6. Application of the assay method

The analytical procedure described here was applied to determine Bobel-24 concentrations in differ-

Table 1
Results of linearity

	r		*a*		*b*		*n*
	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)	
Dog plasma	0.9985	0.10	0.0029	50.7	0.0059	1.9	3
Horse plasma	0.9979	0.06	−0.0380	59.8	0.0810	7.0	3
Horse urine	0.9985	0.15	0.0254	32.9	0.0875	7.3	3
Horse synovial	0.9988	0.05	0.0099	13.2	0.0974	0.1	2

C.V. (%)=coefficient of variation. *a*=*y*-intercept; *b*=curve slope.

ent samples from horses and dogs, for pharmacokinetic studies, metabolic assays and distribution profiles. Plasma concentrations versus time (0, 5, 10, 15, and 30 min, 1, 2, 4, 8, 12 and 24 h), after intravenous and oral administration of 5 mg/kg Bobel-24 in a dog are shown in Fig. 4.

Table 2
Validation of analytical method

	Expected concentration (μg/ml)	Found concentration (mean±S.D.)	Accuracy (%) Inter-assay	Precision (C.V., %)	
				Intra-assay	Inter-assay
Dog plasma		(*n*=12)	(*n*=12)	(*n*=6)	(*n*=12)
	0.5	0.52±0.05	4.0	5.2	8.7
	1.0	1.02±0.02	2.0	11.1	11.1
	5.0	4.87±0.20	−2.6	1.1	4.1
	10.0	10.10±0.67	1.0	4.3	6.7
	20.0	20.00±0.47	0.1	2.5	2.3
Horse plasma		(*n*=18)	(*n*=18)	(*n*=6)	(*n*=18)
	0.625	0.75±0.12	20	9.5	16.1
	1.25	1.32±0.16	5.6	6.2	12.2
	2.5	2.44±0.25	−2.4	9.3	10.2
	5.0	4.86±0.40	−2.8	3.0	8.3
	10.0	9.96±0.45	−0.4	1.7	4.5
	20.0	19.98±0.88	−0.1	4.1	4.4
Horse urine		(*n*=9)	(*n*=9)	(*n*=3)	(*n*=9)
	0.1	0.09±0.01	−10.0	7.1	8.2
	0.5	0.51±0.04	2.0	7.2	7.8
	1.0	0.98±0.06	−2.0	8.2	6.0
	5.0	4.66±0.10	−6.8	5.4	6.9
	10.0	10.3±0.30	3.0	2.6	2.9
	20.0	19.6±0.75	−2.0	2.6	3.8
Horse synovial		(*n*=6)	(*n*=6)	(*n*=3)	(*n*=6)
	0.5	0.55±0.12	10.0	16.3	21.4
	1.0	1.05±0.06	5.0	3.7	7.0
	2.5	2.43±0.21	−2.8	5.5	8.5
	5.0	4.88±0.06	−2.4	0.9	1.2
	10.0	9.98±0.26	−0.2	1.8	2.7

S.D.=Standard deviation.

Table 3
Results of quality control

	Level concentration	Mean±S.D.	C.V. (%)	*n*
Dog plasma	Low	0.75±0.096	12.6	7
	Medium	5.83±0.250	4.3	7
	High	10.61±0.238	9.4	7
Horse plasma	Low	0.91±0.025	2.7	7
	Medium	5.72±0.150	2.5	7
	High	9.41±0.718	7.6	7
Horse synovial	Medium	2.69±0.185	6.9	7

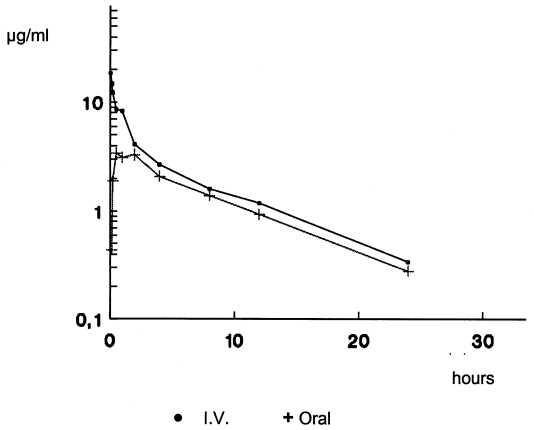

Fig. 4. Dog plasma curve after intravenous administration of 5 mg/kg Bobel-24 and dog plasma curve after oral administration of 5 mg/kg Bobel-24.

The method was also applied to quantify Bobel-24 in pharmaceutical formulations. Bobel-24 and its degradation product were determined for stability studies. Fig. 5. shows a chromatogram from a dermatological preparation of Bobel-24 submitted to 50°C over 15 days, for accelerated stability testing. The concentration of 2,6-diiodophenol, the degradation product in this sample was estimated as 0.1% of Bobel-24 concentration.

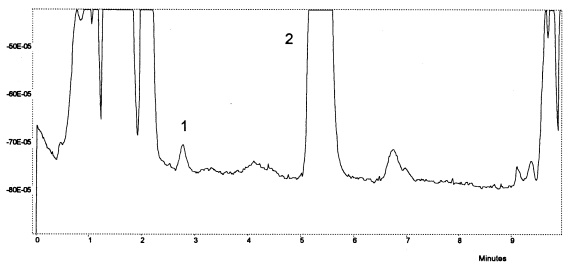

Fig. 5. Chromatogram of a Bobel-24 dermatological sample after 15 days at 50°C (peak 1=2,6-diiodophenol; peak 2=Bobel-24).

4. Discussion

The present method has proven suitable for use in pharmacokinetic studies and tissue distribution profiles in rats, dogs and horses. It can be applied to metabolic studies as urine samples were analyzed by the same method. It is also useful for QC assays and stability studies of pharmaceutical formulations of Bobel-24. This method is stability indicating, as degradation products can also be detected.

Bobel-24 was stable in plasma and homogenized tissue for at least three months when stored at −40°C. Urine samples were not stable as other interfering peaks were observed. Unless urine samples are analyzed on day they are obtained, a stabilizer is necessary in frozen samples.

This is a versatile HPLC method for quantitative determination of a new antiinflammatory drug and has been successfully used in biological samples and pharmaceutical preparations for research studies.

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