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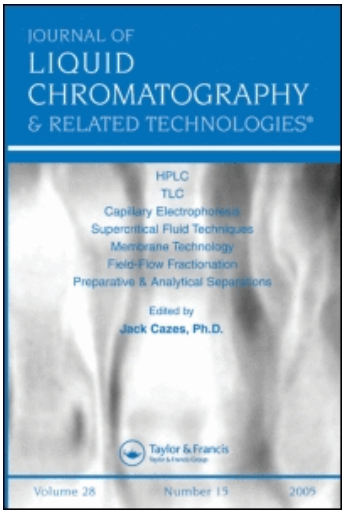

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Analysis of Bunaprolast, An Esterase Unstable Drug, with An Active Metabolite Subject to Oxidative Degradation, in Blood Plasma

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Original Article

ANALYSIS OF BUNAPROLAST, AN ESTERASE UNSTABLE DRUG, WITH AN ACTIVE METABOLITE SUBJECT TO OXIDATIVE DEGRADATION, IN BLOOD PLASMA

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ABSTRACT

This report describes a sensitive, selective and robust assay for bunaprolast and an active metabolite in canine and human plasma. Method development was complicated in that bunaprolast is quickly hydrolysed by esterases even in vitro, and the metabolite is rapidly oxidised in dilute aqueous solutions. Effective measures to stabilize analytes in biological matrices and during sample extraction are described.

The method involves the selective solid phase extraction of analytes with a close analogue as internal standard followed by reversed phase HPLC and fluorescence detection. The limit of quantification was typically less than 1 ng/ml, and the assay was linear over the range 1 - 1,000 ng/ml.

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INTRODUCTION

Bunaprolast (Upjohn compound U-66,858), is an inhibitor of leukotriene biosynthesis. Its design is such that it is quickly modified by endogenous esterases to an active metabolite (Upjohn compound U-68,244). A sensitive assay for both bunaprolast and U-68,244 in blood plasma was required to support animal (pharmacokinetic and toxicology) studies and human volunteer clinical trials.

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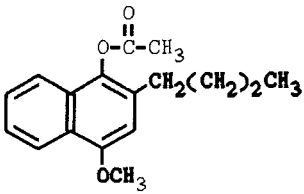
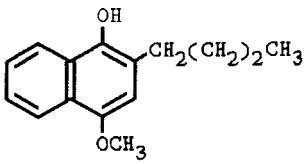
The development of high pressure liquid chromatographic assays was complicated by (a) the half life of bunaprolast is very short in whole blood and plasma in vitro, (b) U-68,244 is readily oxidised in whole blood and dilute solutions to unidentified products. Strategies had to be devised to minimise the loss of bunaprolast during blood sampling and sample storage, and to prevent oxidation of U-68,244 during sample extraction and measurement. The analytical problem was compounded by the need for routine application of the assay and the low levels (single ng/ml or less) of both analytes that had to be quantified.

Blood samples were collected into tubes containing an esterase inhibitor and plasma was prepared rapidly. Samples from dog and man could be safely stored deep frozen. Samples were prepared for analysis by solid phase extraction in the presence of a strong reducing agent. Bunaprolast and U-68,244 were analysed by isocratic HPLC and fluorescence detection with wavelength programming, which enabled optimum sensitivity for all analytes to be achieved.



U-66,858



U-68,244

Figure 1 Structures of drug and metabolite.

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MATERIALS

Chemicals and reagents

Bunaprolast, 1-naphthalenol 2-butyl-4-methoxy acetate, the hydroxylated metabolite U-68,244 (fig 1) and a methyl analogue of bunaprolast (for use as an internal standard) were obtained from the Upjohn Company, Kalamazoo, USA. Sodium acetate, glacial acetic acid, ascorbic acid, sodium fluoride, acetonitrile, methanol and water were all obtained from Fisons, Loughborough, UK, and were of AR or HPLC grade. A selection of reagents with antiesterase activity were obtained from Sigma, Poole, Dorset, UK.

Equipment

Analytichem Bond Elut CBA cartridges (100 mg) and Vac-Elut ten place manifolds were obtained from Jones

Chromatography (Hengoed, Mid Glammorgan UK). A Denley bench top centrifuge, model 402 (Denley, Billingshurst, Sussex, UK) with appropriate mini vials was used for preparation of plasma samples.

METHODS

Preparation of standard solutions for calibration curve and internal standard

Bunaprolast and U-68,244, were dissolved in acetonitrile to give solutions of approximately 1 mg/ml. Internal standard solutions were similarly prepared at a concentration of 0.1 mg/ml. Aliquots of these were diluted in acetonitrile to give solutions in the range 0.25-10 μg/ml for spiking aliquots of control plasma. Dilute solutions were unstable even at -20 °C and were prepared weekly.

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Treatment of blood samples

Blood samples (2-5 ml) from human or dog were collected in polypropylene tubes containing EDTA and 5 mg of sodium fluoride (NaF) coated to the wall of the tube. Samples were agitated to ensure dissolution of NaF and plasma was prepared as quickly as possible by centrifugation. Plasma was frozen and stored at -20 °C before analysis.

Preparation of plasma for analysis

Aliquots (250 µl) of thawed plasma were transferred to 1 ml centrifuge tubes and 25 µl of internal standard solution (approximately 200 µg/ml) added. After thorough mixing 750 µl of acetonitrile were added. Tubes were mixed and centrifuged at 12,000 rpm for 2 minutes. The supernatants were transferred to glass tubes containing 2 ml of 0.05 M ascorbic acid solution. The centrifuge tubes and protein pellet were rinsed with a further one ml of 0.05 M ascorbic acid solution, which was added to the tube contents. The prepared solutions were extracted immediately.

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Preparation of calibration samples

Calibration samples were prepared over the range of, typically, 1-100 ng/ml for both bunaprolast and the metabolite U-68,244. These were prepared daily by adding up to 50 µl each of standard solutions to 1 ml of " control plasma" containing 5 mg/ml NaF. After mixing , 250 µl aliquots were prepared as for experimental samples.

Extraction

The required number of Vac Elut boxes were loaded with Bond Elut CBA (carboxylic acid phase) cartridges (100 mg, 1 ml size). Cartridges were primed successively with 2 ml acetonitrile, 2 ml of the eluting solution (40% acetonitrile, 30% methanol,

made up to 100% (v/v) with 0.05 M ascorbic acid solution) , and 2 ml of 0.05 M ascorbic acid solution. The prepared plasma samples were drawn slowly through the cartridges immediately after priming. Cartridges were then rinsed with 2 ml of 0.05 M ascorbic acid solution followed by 2 ml of 15% (v/v) acetonitrile in 0.05 M ascorbic acid solution. Analytes were desorbed from the cartridges by 600 μl of eluting solution. The vacuum in the Vac Elut was maintained until the eluates had been partially evaporated to approximately 200 μl.

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HPLC Instrumentation and Chromatographic conditions

The HPLC system consisted of a Waters 510 twin piston pump, Waters WISP 712 autoinjector with low volume vials (Millipore Ltd, Milton Keynes, U.K.), a Shimadzu CTO-6A column oven (Dyson Instruments, Hatton, U.K.) and a Perkin Elmer LS 4 fluorescence detector (Perkin Elmer Ltd, Watford, U.K.). Data were captured on a Spectra Physics SP 4270 integrator/recorder (Spectra Physics Ltd, Hemel Hempstead, U.K.).

The HPLC column was a Dupont Zorbax phenyl , 250 x 4.6 mm i.d.(Hichrom Ltd, Reading, U.K.) maintained at 40 °C. The mobile phase consisted of 55% (v/v) acetonitrile with 45 % 0.01 M sodium acetate solution adjusted to pH 4.5 with glacial acetic acid, degassed with helium before use. The flow rate was 2 ml/min. The fluorescence detector was set at an excitation wavelength of 237 nm throughout a chromatographic run, whilst the emission wavelength was set at 410 nm at

the beginning of a run (optimum for U-68,244) and switched to 380 nm (optimum for bunaprolast and the internal standard) seven minutes into the chromatographic run. The injection volume was 50-150 μl and the total run time 13-15 minutes.

The SP4270 recorder/integrator was configured to record peak height ratios of bunaprolast: internal standard and U-68,244: internal standard. Data were downloaded to a mainframe computer via a Spectra Physics Chromstation AT (Spectra Physics, Hemel Hempstead, U.K.). Further statistical analysis was carried out using the computer program SAS (SAS Institute Inc., North Carolina, USA). Linear regression was carried out using peak height ratio versus concentration using a weighted function, 1/concentration squared.

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RESULTS AND DISCUSSION

HPLC

Bunaprolast is a relatively non-polar compound that exhibited good chromatographic characteristics with a number of hydrophobic and polar stationary phases and acetonitrile/water mobile phases containing a small proportion of trifluoroacetic acid (TFA). Typically, the addition of 0.1-0.2% TFA increased retention of bunaprolast and the internal standard (K' increased from two to about five) on Zorbax nitrile (CN) whilst slightly worsening peak symmetry. Good results were

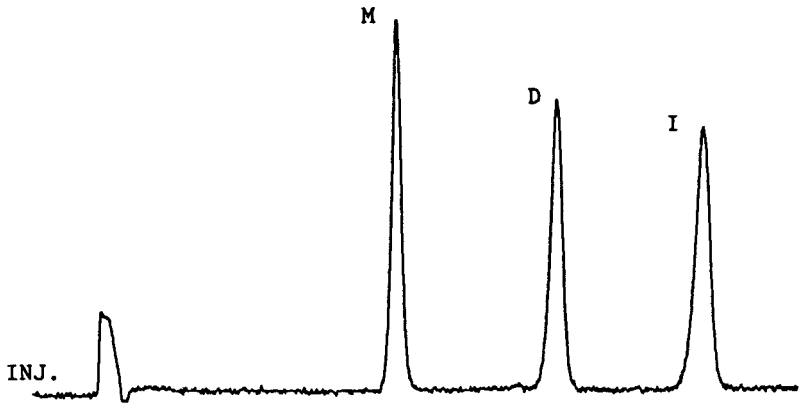

Figure 2 Chromatogram of standard solutions approximately 10 ng on column using detector wavelength programming. M = U-68,244, D = U-66,858 and I = internal standard. Total run time for all chromatograms was 15 minutes.

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also obtained with C8 and C18 silica bonded phases, which also completely resolved the more polar metabolite U-68,244 from bunaprolast, but Zorbax phenyl gave good efficiency (10,000 to 14,000 theoretical plates per column, as measured with bunaprolast) and gave good selectivity (fig II). This system was used to support initial animal experiments, and had the advantage that plasma samples could be directly injected on column without degradation in performance. Replacing TFA in the mobile phase with acetate buffer did not degrade chromatography, and resulted in better selectivity for biofluid extracts, minimising the solvent front. Fluorescence detection was more sensitive than UV detection, and about 100 pg on column could be successfully integrated. In

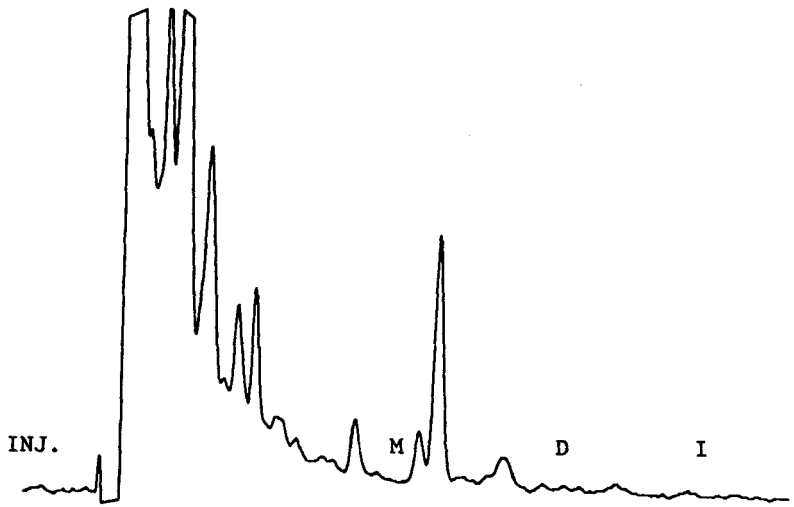

Figure 3 Control dog plasma, 250 μl sample.

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practice the limit of quantification of bunaprolast and to a lesser extent U-68,244 was sometimes limited by endogenous interferences. Dog plasma extracts were essentially free of peaks running with or close to analytes (fig III and IV). The effect of wavelength programming also results in better selectivity and sensitivity (fig V). Some samples of control human plasma showed a small peak coeluting with bunaprolast. This represented a concentration of about 0.5 ng/ml at most and was not a problem in practice (fig VI).

Stability of analytes

Bunaprolast was unstable in blood and plasma, being converted in vivo and in vitro to U-68,244. This can

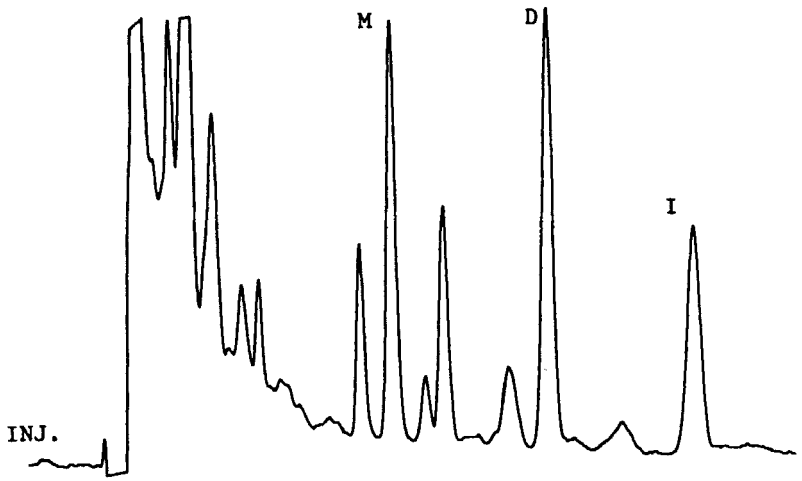

Figure 4 Control dog plasma spiked with 25 ng/ml U-68,244 and 10 ng/ml U-66,858. Chromatogram from 250 μl of sample without detector wavelength programming.

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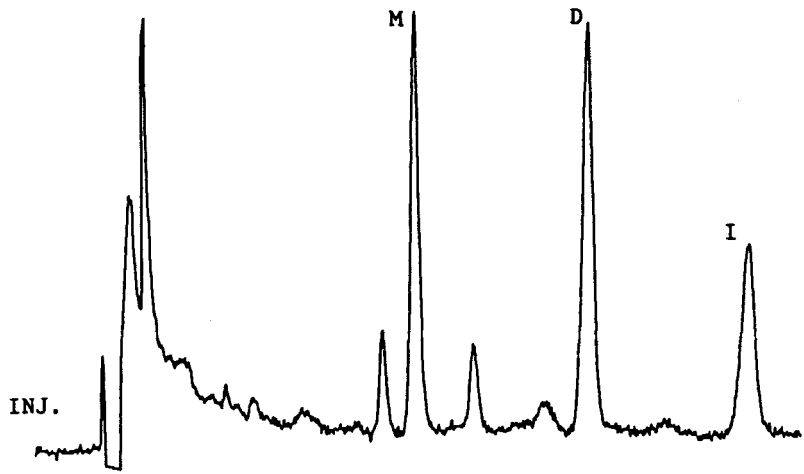

Figure 5 Human standard spiked at 25 ng/ml of U-68,244 and U-66,858. Chromatogram from 250 μl of sample using detector wavelength programming.

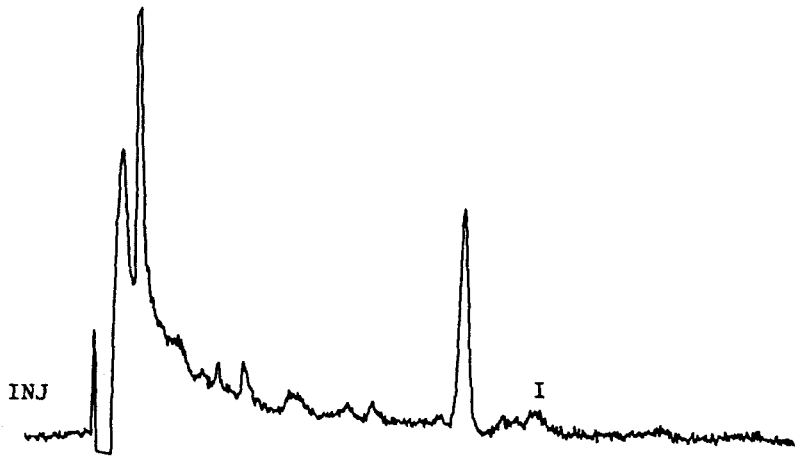

Figure 6 Human control plasma (500 μl sample) showing interfering peak, I at the U-66,858 retention time.

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effectively inhibited in human or dog plasma by the addition of sodium or potassium fluoride to sample tubes. The concentration used (approximately 5 mg/ml) was sufficient for this and did not cause gross lysing of red cells. Samples stored at -20 °C were stable for at least a month.

Not unexpectedly this procedure was not sufficient to control loss of U-66,858 and the internal standard from rat plasma. To reduce hydrolysis to a minimum rat plasma had to be rapidly deproteinated with acetonitrile. The process from bleeding the rat to the prepared plasma being frozen was carried out in under 2 minutes. Deproteination of whole blood was not a successful alternative and loss of U-68,244 was seen.

Other antiesterase agents were tried, such as ethyl pyrophosphate, neostigmine, and phyostigmine; these appeared less effective than sodium fluoride The specific esterase inhibitor phenyl methyl sulphonyl chloride did give stable biofluid samples, but was considered too toxic for routine use.

In practice, no variations due to the hydrolysis of bunaprolast or of the internal standard was seen with the precautions described.

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U-68,244 was unstable in whole blood and in dilute aqueous solution, and was readily oxidised to an unknown product. The excess of ascorbic acid present during sample preparation, extraction, and in the cartridge eluate was effective in minimising this. Other strong reducing reagents, such as sodium metabisulphite would probably been equally effective, but their presence in extracts would cause a degradation of HPLC performance.

Some loss of U-68,244 was seen in study samples during freeze/thaw cycles (as opposed to spiked control samples) and samples were therefore divided before storage. Extracts were stable and could be reinjected onto the HPLC after 12 hr in an auto injector without loss of analyte or the appearance of interfering peaks.

Extraction

The extraction of bunaprolast, U-68,244 and internal standard was adequate and reproducible but was

TABLE I

Calibration curve for U-68,244 and U-66,858 in human control plasma.

Concentration ng/ml (U-68 /U-66)	peak height ratio U-68	CV (%)	peak height ratio U-66	CV (%)
0.95/4.76	0.078		0.552	
	0.065	9.37	0.474	8.20
	0.075		0.489	
	0.942		0.985	
9.52/9.52	0.927	0.99	0.900	4.75
	0.944		0.969	
	2.106		2.291	
23.57/23.81	2.147	2.01	2.279	3.96
	2.235		2.132	
	5.279		4.425	
47.14/47.62	5.469	4.06	4.354	2.87
	5.042		4.184	
	6.604		5.466	
59.05/59.52	6.575	0.36	5.392	1.44
	6.575		5.311	
	10.133		9.195	
94.29/95.24	9.990	0.76	8.633	3.06
	10.110		8.827	
	26.518		20.992	
235.95/237.86	25.800	2.01	20.39	1.91
	25.502		20.256	
	54.703		45.261	
471.91/475.71	55.761	2.42	46.463	6.18
	57.386		50.842	
r	0.9971		0.9974	
slope	0.1002		0.0915	
intercept	-0.0311		0.0697	
	(0.03 ng/ml)		(-0.76 ng/ml)	

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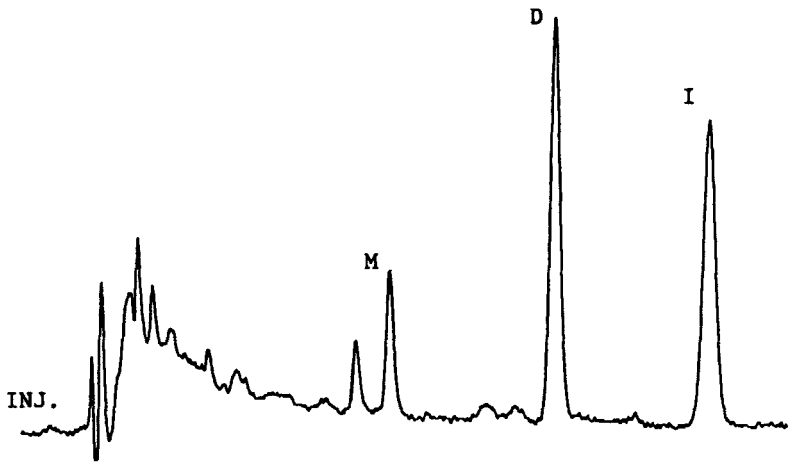

Figure 7 Control rat plasma spiked with 100 ng/ml of both U-68,244 and U-66,858. Sample size was 50 μl. Detector wavelength programming was not used.

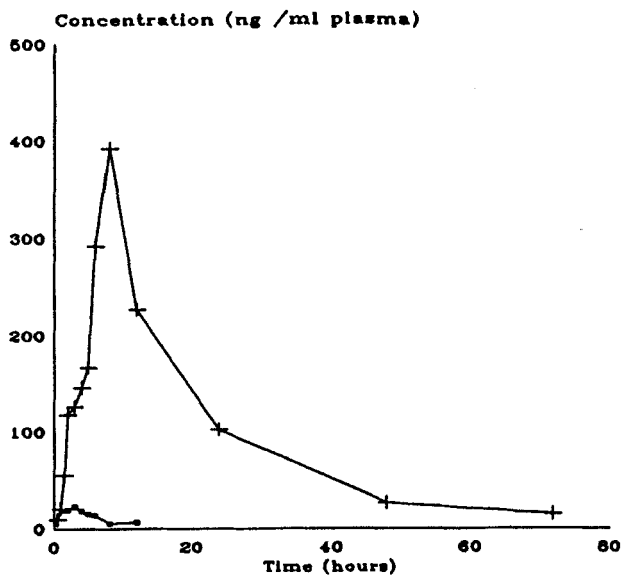

Figure 8 Plasma profiles for U-66,858 (-O-) and U,68,244 (-X-) in dog after a 1000 mg oral dose of Bunaprolast.

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dependent, in part, upon the dissociation of analytes from plasma protein, either by addition of acetonitrile (as described in the text) or by an equally effective tenfold dilution of samples with 0.5 M ascorbic acid solution. Addition of other disrupting agents, for example sodium dodecyl sulphate, trichloroacetic acid and Triton X-100 was less effective, and a loss of up to 40% through the cartridge was seen. Addition of acetonitrile and dilution of the sample to 4 ml was convenient and effective ; an extraction efficiency of at least 75% at all concentrations was routinely achieved.

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Linearity and Limit of Quantification

The assay was linear in the range 1-500 ng/ml for both bunaprolast and U-68,244 in dog plasma and in the ranges 1-100 ng/ml (U-68,244) and 5-500 ng/ml (bunaprolast) in human plasma, with coefficients of regression (r) of 0.99 or better (Table I). A concentration of 1 ng/ml of both analytes could be quantified with good precision in dog plasma, but an interfering peak limited quantification of bunaprolast to 5 ng/ml in human plasma, but U-68,244 was not affected.

DISCUSSION

This method has been used routinely for supporting large toxicological studies, showing that the stability problems associated with assay of this

compound and its metabolite have been successfully overcome (fig VII). It is suggested that the strategies followed have general application for drugs and metabolites that show similar characteristics. The use of solid phase extraction and HPLC on a contrasting stationary phase results in a very "clean" extract, the whole of which can be injected on column without compromising chromatographic performance. The procedure has also been applied to other biofluids, for example from the cannulated rat (fig VIII), where a limit of quantification of 25 ng/ml for 50 μl plasma samples was achieved and 2 ml samples of lung lavage from an intra-venously dosed dog was successfully extracted and analysed. The presence of bunaprolast was confirmed by GC-MS.

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