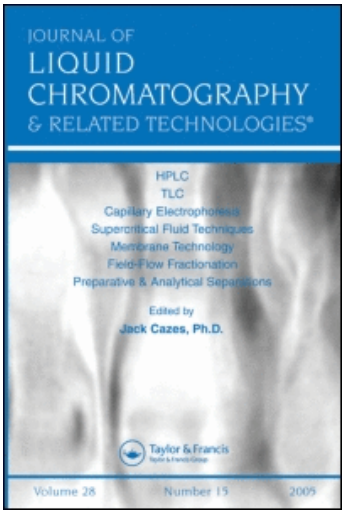

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Original Article

AN HPLC ASSAY FOR A PROSTACYCLIN ANALOGUE, CIPROSTENE CALCIUM, IN HUMAN PLASMA

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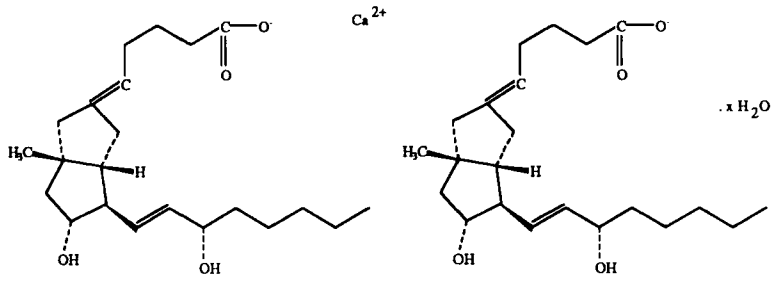
ABSTRACT

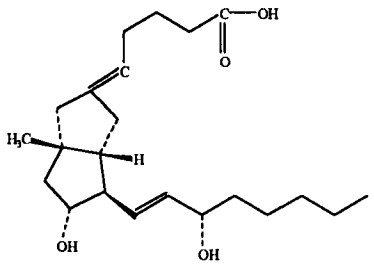
A sensitive assay has been developed for the quantification of the prostacyclin analogue, ciprostene calcium, in human plasma. The method involves solid phase extraction of ciprostene calcium and internal standard, carbacyclin, from a small volume of human plasma. The extract is derivatized with 4-bromomethyl-7-acetoxycoumarin, and the derivatized product extracted with a polar solid phase cartridge and concentrated by evaporation. The final extract is separated by reversed phase HPLC and measured by a fluorimetric detector following post-column alkaline hydrolysis. The overall extraction efficiency is better than 75%, and the assay is linear over the concentration range studied (5-20 ng/ml). The limit of quantification was approximately 5 ng/ml. Ultimate sensitivity was limited by interfering peaks endogenous to the biological matrix. Coefficients of variation at mid-range concentrations are less than 10%.

INTRODUCTION

Ciprostene calcium ("ciprostene", Fig 1) a chemically stable analogue of prostacyclin, is intended as an inhibitor of



Ciprostene calcium



Carbacylin

FIGURE 1

Structure of ciprostene calcium and carbacyclin.

platelet aggregation. Though sensitive methods exist to measure endogenous prostaglandins in biofluids, such methods involving complex sample preparation (1) and GC-MS (2) are not suitable for routine application to large numbers of samples. Measurement of endogenous or synthetic prostaglandins in biological fluids by HPLC is difficult due to the absence of a suitable chromophore, fluorophore or electrochemically active

group in this class of molecule, and the very low concentrations (single ng/ml or lower) expected in biofluid. Derivatization is therefore necessary to achieve an HPLC assay of the required sensitivity. A number of reagents that react with the carboxylic acid group of prostaglandins to yield a UV-absorbing or fluorescent compound have been reported (3-9). Fluorescence techniques offer the best chance of achieving high sensitivity together with a robust detection technique, although derivatives that are chemiluminescent (10) or electrochemically active (11) have also been demonstrated.

The derivatizing agent 4-bromomethyl-7-acetoxycoumarin (BrMac) (3) has been used in the development of this assay as it offers a high fluorescence yield, is stable both in solid form and solution, and is freely available commercially. The product is only fluorescent following alkaline hydrolysis at 80°C in a post-column reaction system (PCRS), and although this slightly complicates the HPLC system, it has the advantage that the fluorescent yield is relatively unaffected by the nature of the carboxylic acid or by the composition of the HPLC mobile phase.

The method presented is straightforward, robust and capable of routine application, and offers sensitivity to at least 5 ng/ml. Sensitivity is currently limited not by the detection system or the nature of the derivative, but by interfering substances endogenous to plasma which are co-extracted and derivatized.

EXPERIMENTAL

Materials

Ciprostene (ciprostene calcium; (5Z)-9ß-methyl-6a-carbaprostaglandin I_2, calcium salt) and carbacyclin ((5E)-6a-carbaprostaglandin I_2) were supplied by The Upjohn Company (Kalamazoo, Michigan, U.S.A.). Trifluoroacetic acid,

acetonitrile (far UV grade), methanol, methyl tertiary butyl ether (MTBE), n-hexane, ethyl acetate, dibenzo-18-crown-6 ether (reagent grade), potassium hydrogen carbonate (A.R. grade), sodium sulphate (A.R. grade) were all supplied by Fisons Ltd (Loughborough, U.K.) and were of HPLC grade unless indicated otherwise. Acetone (spectrophotometric grade) and 7-acetoxy-4-(bromomethyl)-coumarin were obtained from Aldrich Ltd (Gillingham, Dorset U.K.). Sodium hydroxide and orthophosphoric acid were both of AnalaR grade and obtained from BDH Ltd (Poole, Dorset, U.K.). Reagent grade water was produced as required in-house by a Millipore Milli-Q system (Millipore Ltd, Watford, Hertfordshire, U.K.).

Equipment for Extraction and Derivatization

Analytichem Bond Elut C2 and CN 100mg/1ml extraction cartridges and SPS 24 VAC ELUT vacuum manifolds were obtained from Jones Chromatography (Hengoed, Mid Glamorgan, UK). Techne SC-3 Sample Concentrator was purchased from Techne Ltd. (Cambridge, UK), and the Pierce Reacti-Therm heating/stirring unit, Reacti-Vials, 0.3 ml size with appropriate caps, seals and spin vanes from Life Science Laboratories Ltd (Luton, Bedfordshire, UK).

Preparation of Control Plasma and Calibration Standards

Plasma for preparation of spiked calibration standards was collected using plastic syringes and containers with EDTA as anti-coagulant. The plasma was separated and stored at -20°C. After thawing, samples of plasma with evidence of particulate or coagulated material were centrifuged at 12,000g for 2 minutes. Only clear biofluid was extracted.

Stock solutions of ciprostene and carbacyclin were prepared at a concentration of 100 μg/ml in methanol. These solutions

were diluted further with methanol to give standard working solutions. Aliquots (1 ml) of control plasma were spiked with ciprostene to give calibration standards over the range of 5-20 ng/ml.

Plasma Extraction

The required number of Bond Elut C2 cartridges (100 mg/1ml size) were loaded onto SPS 24 Vac Elut boxes. Cartridges were primed successively with 2 x 1ml CH_3CN, 1ml H_2O, and 1ml 1% phosphoric acid solution using a minimum of applied vacuum at each stage. Cartridges were not allowed to dry out after priming. Plasma samples (0.25ml) were spiked with 50 μl 0.375 μg/ml carbacyclin internal standard solution, and diluted with 0.75 ml 1% phosphoric acid prior to loading onto the cartridges.

The primed cartridges were loaded with the prepared plasma and the samples were drawn through with a minimum of vacuum. Cartridges were washed successively with 2 x 1ml H_2O and 2 x 1ml 40% MeOH, and then dried by maintaining full vacuum (30 mmHg on dial) to the extraction boxes for at least 10 minutes. The cartridges were then washed twice with 1 ml 25% MTBE, 75% n-hexane and eluted with 1 ml of a mixture of 80% MTBE, 20% n-hexane. The vacuum in the extraction station was maintained until the eluting solvent had completely evaporated. All test tubes used were polypropylene (Sarstedt Ltd, Leicester, U.K.) and all liquid transfers were performed with polypropylene Pastettes (Alpha Laboratories Ltd, Eastleigh, U.K.) or Gilson Pipetman pipettes (Anachem Ltd, Luton, U.K.).

Derivatization

Extracts were redissolved in 100 μl methanol, transferred to 0.3 ml Reacti-Vials, and the methanol evaporated with a

gentle stream of air. A 1:1 mix of solid $KHCO_3$ and Na_2SO_4 (10 mg) was added to each vial together with a spin vane, 50 μl of 200 nmol/ml solution of dibenzo-18-crown-6 ether in acetone and 50 μl of a 1 μmol/ml solution of 4-bromomethyl-7-acetoxycoumarin in acetone. The tubes were capped and heated at 50°C with mixing for 30 minutes in the Reacti-Therm unit. Each tube was checked to ensure that the spin vane rotated freely.

After cooling the reaction mixtures were transferred to 12 x 65 mm polypropylene tubes using fine tip polyethylene Pastettes, 900 μl of n-hexane added, and the tubes vortex mixed. Bond Elut CN cartridges were primed with two 1 ml aliquots of n-hexane, and the diluted derivatized extracts drawn through the cartridges with the minimum of applied vacuum; the cartridges were then washed twice with 1 ml n-hexane, and twice with 1 ml of 20% ethyl acetate, 80% n-hexane.

The cartridges were partially dried by maintaining full vacuum (20-25 mm Hg on gauge) for 1 minute, and then eluted slowly with 1 ml of CH_3CN. The solvent was evaporated with a stream of air in a Techne Sample Evaporator set at 30°C, and the residue redissolved in 150 μl of 50% CH_3CN, 0.1% TFA.

HPLC Instrumentation and Chromatographic Conditions

The HPLC system consisted of a Waters 600 twin piston pump, Waters WISP 712 autoinjector (Millipore Ltd, Milton Keynes, U.K.), a Shimadzu CTO-6A column oven (Dyson Instruments, Hetton, U.K.), and a Perkin-Elmer LS-40 Luminescence Detector (Perkin-Elmer, Beaconsfield, U.K.). Chromatograms were recorded on a Spectra Physics SP4270 integrator/recorder (Spectra-Physics, Hemel Hempstead, U.K.) and raw chromatographic data captured by a Spectra Physics Chromstation data system. A Dupont Zorbax ODS HPLC column, 5 m, 25cm x 4.6mm (HiChrom Ltd, Reading, U.K.), maintained at 45°C, was used with a Brownlee

NewGuard RP18, 15 x 3.2 mm guard column (Anachem Ltd., Luton, U.K.). The mobile phase consisted of acetonitrile-water-TFA (55:44.9:0.1 by volume) pumped at 1.5 ml/min. Fluorescence was monitored using a Perkin Elmer LS-40 detector (Beaconsfield, Bucks, U.K.) with an excitation wavelength of 370 nm and an emission wavelength of 466 nm.

Post Column Reaction

Post column hydrolysis of analytes was performed with a Kratos PCRS 520 Heating unit equipped with two 2ml knitted teflon reaction coils in series. The hydrolysis reagent, 0.1 M sodium hydroxide, was pumped by a Severn Analytical SA6410B Pump (Severn Analytical, Shefford, U.K.) set at a flow rate of 0.5 ml/min. The PCRS reaction coils were heated to 80°C.

RESULTS AND DISCUSSION

Extraction And Derivatization

The selectivity of the initial extraction from plasma was optimized using the C2 extraction cartridge in two modes, first reversed-phase, and second, after drying the cartridges, using solvents favouring polar interactions. A further polar cartridge extraction following derivatization was primarily to clean-up by-products of the reaction, although this will also contribute some further selectivity when extracting plasma. Extraction efficiency for ciprostene from plasma was estimated by a comparison of peaks given by unextracted derivatized standard and derivatized standards extracted from plasma, and was between 75% and 80% (78.2±8.5%, n=3, 20 ng/ml ciprostene).

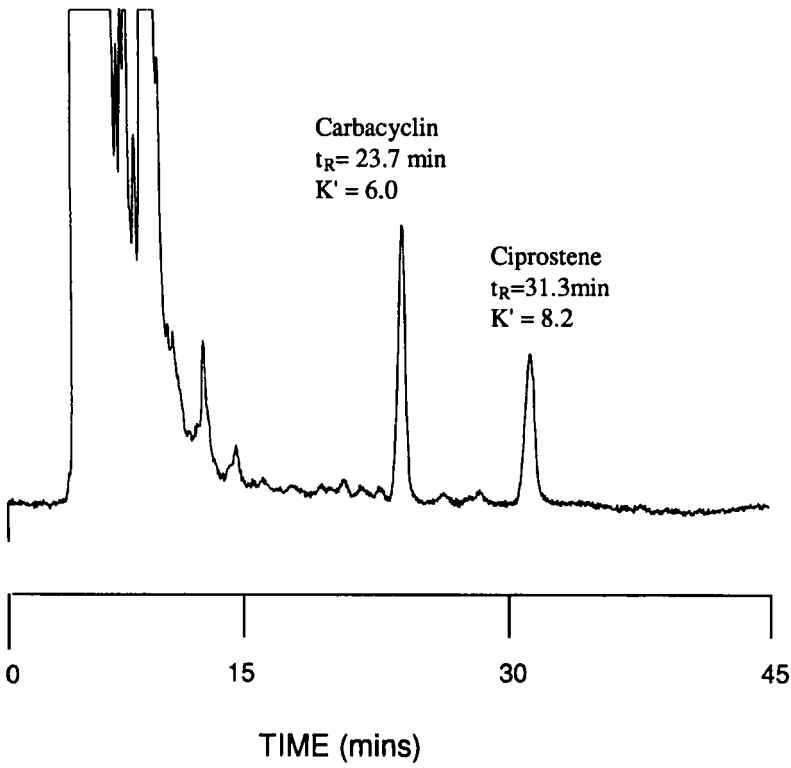


FIGURE 2

HPLC trace of an injection of derivatized standard solution containing 1 ng ciprostene calcium and 1.5 ng of carbacyclin, (conditions as text).

HPLC

Reversed phase HPLC was chosen because of its inherent flexibility for analysing complex mixtures. Ciprostene was eluted with reasonable peak shape giving a typical system effciciency of N=14,250 (half height method, Fig. 2) and skew of 1.2, in spite of the increased dead volume due to the post

column reaction system. A relatively high retention of analytes was required to obtain optimum selectivity. Other bonded silica phases (e.g. phenyl, CN, TMS and C8) were investigated, but did not separate the analyte peak from large peaks of an endogenous origin. Under best conditions, the equivalent of approximately 50 pg of derivatized ciprostene standard could be detected on column (S/N 1:3). In practice the limit of detection was compromised by peaks of endogenous origin in extracts, which limited the sensitivity of the assay to 5 ng/ml ciprostene inhuman plasma (Fig. 3, 4 & 5). The potential of this method is indicated by the high on column sensitivity of the derivative; further development to fulfill this potential will require a more specific extraction procedure, such as could be provided by immunoaffinity extraction methods (1,12). A small endogenous peak underlies the internal standard but the effect of this is minimised by using a relatively high concentration of carbacyclin (75 ng/ml). Improved recovery and precision for the assay were noted on increasing the concentration of internal standard, indicating that a high concentration of carbacyclin may act as a "carrier" and reduce losses of ciprostene during the extraction and derivatization procedures.

Linearity

Calibration lines were linear over the range of 5 ng/ml to 20 ng/ml. The coefficient of linear regression (r) using a weight of $1/x^2$ was 0.98 or better.

Repeatability

Coefficients of variation for triplicate analysis of spiked control human plasma are shown in Table 1. Satisfactory precision of analysis was shown at all concentrations studied.

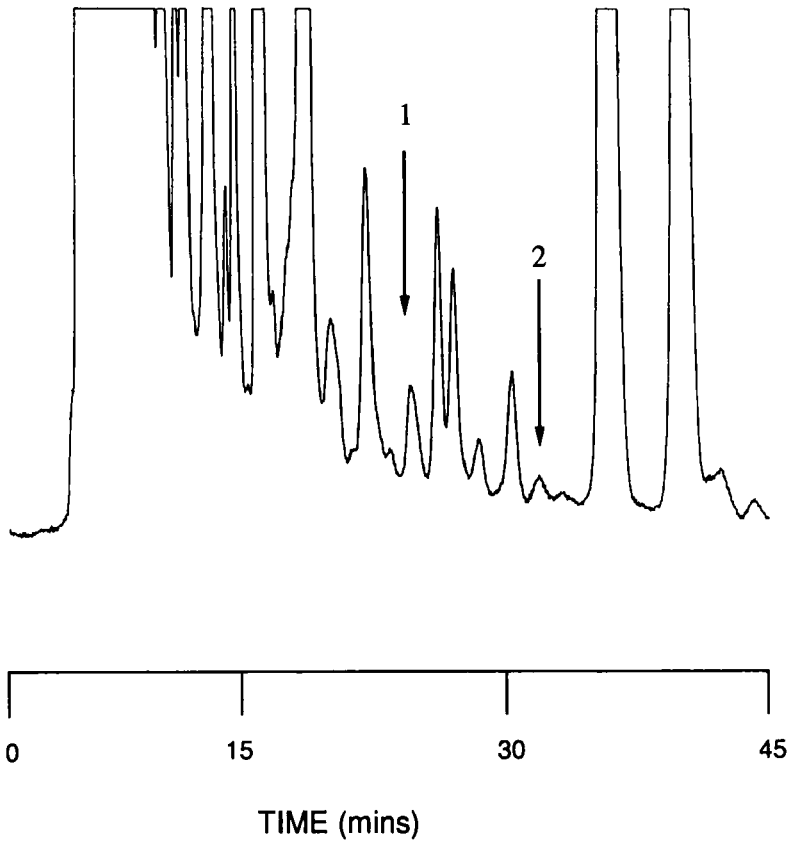


FIGURE 3

HPLC trace of an extract of blank control human plasma , (conditions as text). Arrows indicate positions of carbacyclin (1) and ciprostene (2).

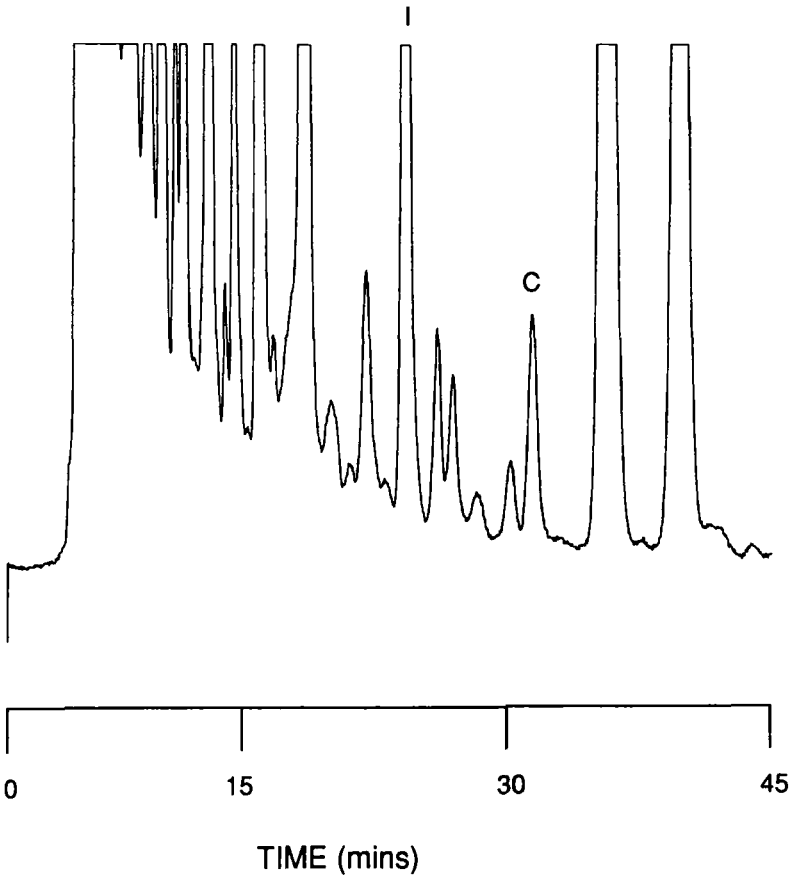


FIGURE 4

HPLC trace of an extract of control human plasma spiked with 20 ng/ml of ciprostene calcium (C) and 75 ng/ml of carbacyclin (I), (conditions as text).

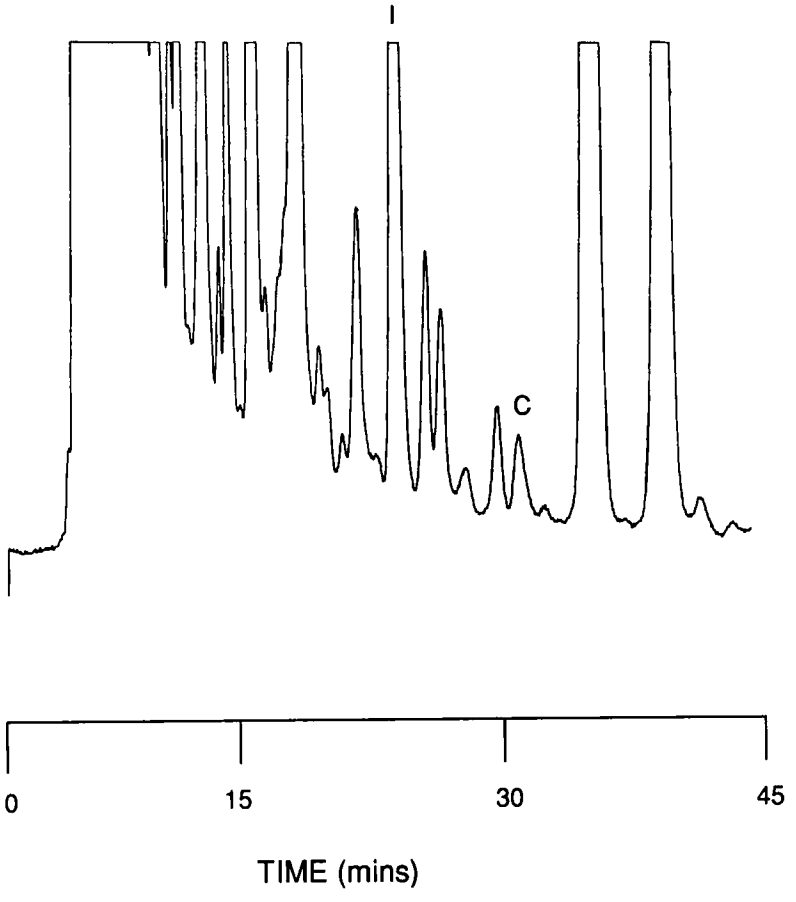


FIGURE 5

HPLC trace of an extract of control human plasma spiked with 5 ng/ml of ciprostene calcium (C) and 75 ng/ml carbacyclin (I), (conditions as text).

TABLE 1

Precision Data Over the Concentration Range of 5 to 20 ng/ml Ciprostene Calcium in Control Human Plasma.

Conc. of ciprostene in spiked plasma (ng/ml)	Day 1		Day 2		Day 3		Inter-assay CV%
	Measured conc ciprostene (ng/ml) Mean±S.D.	CV%	Measured conc ciprostene (ng/ml) Mean±S.D.	CV%	Measured conc ciprostene (ng/ml) Mean±S.D.	CV%	
5	5.1±0.5	9.8	5.1±0.15	3.0	5.0±0.38	7.5	6.4
10	9.3±0.85	9.1	9.6±0.17	1.8	9.6±0.92	3.1	6.8
15	14.6±0.47	3.2	14.7±0.23	1.6	15.7±2.6	16.5	9.5
20	21.3±0.8	3.8	20.8±0.52	2.5	19.7±1.0	5.3	4.9

(Day 1: r=0.987, n=12; Day 2: r=0.997, n=12; Day 3: r=0.981, n= 12)
For all intra-assay precision measurements, n=3; for all inter-assay precision measurements, n=9.

Intra- and inter-assay C.V.s are below 10% at concentrations above 5 ng/ml in plasma.

Limit of Quantification

Concentrations down to 5 ng/ml of ciprostene in human plasma could be measured with inter- and intra-assay coefficients of variation of less than 10%. Quantification below this concentration is limited by the appearance of an endogenous peak underlying the ciprostene peak. Interference from extracts of control plasma sample have been similar in samples obtained from a number of different individuals. These interferences may be reduced in extracts from plasma where the plasma has been sampled and prepared differently (14).

CONCLUSION

Despite the complexity of the assay, it is reasonably straightforward in practice, applicable to routine sample analysis, and has sufficient sensitivity to measure steady state concentrations of ciprostene during infusion (40-120 ng/kg/min for 3-8 hours). To follow the full pharmacokinetic profile of this drug, however, a higher sensitivity assay will be required. Development of the assay to its theoretical lowest sensitivity (i.e. 50-100 pg/ml ciprostene from a 1 ml sample) would require either highly complex extraction procedures (13), or the use of novel extraction techniques such as double anti-body precipitation (12) or affinity chromatography (1). Such approaches are currently handicapped by the lack of a specific anti-body for ciprostene.

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