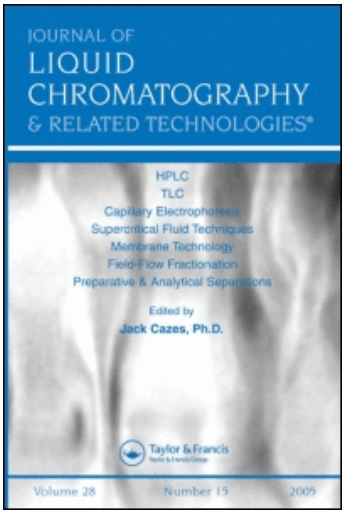

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Evaluation of Electrochemical and Fluorescence Detection in Liquid Chromatography for the Assay of Indomethacin in Aqueous Humour Samples

O. Baudrit[a]; H. Fabre[a]
[a] Laboratoire de Chimie Analytique Faculté de Pharmacie, Montpellier Cédex 1, France

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EVALUATION OF ELECTROCHEMICAL AND FLUORESCENCE DETECTION IN LIQUID CHROMATOGRAPHY FOR THE ASSAY OF INDOMETHACIN IN AQUEOUS HUMOUR SAMPLES

O. BAUDRIT* AND H. FABRE
Laboratoire de Chimie Analytique
Faculté de Pharmacie
F-34060 Montpellier Cédex 1, France

ABSTRACT

The performance of amperometric and fluorescence detection *vs* UV detection in high performance liquid chromatography (HPLC) have been evaluated for monitoring indomethacin at trace levels in rabbit aqueous humour. Amperometric detection at a potential of +1.20V (*vs* Ag/AgCl) did not present any advantage over UV detection. However, fluorescence detection after liquid-liquid extraction with ethyl acetate and pre-column hydrolysis yielded limits of detection (2 $\mu g\ l^{-1}$) and quantitation (5 $\mu g\ l^{-1}$), lowered by a factor of 2 by comparison with UV detection. Validation results for a fluorescence method in aqueous humour are presented concerning specificity, linearity, accuracy, precision, analytical recovery, limits of detection and quantitation.

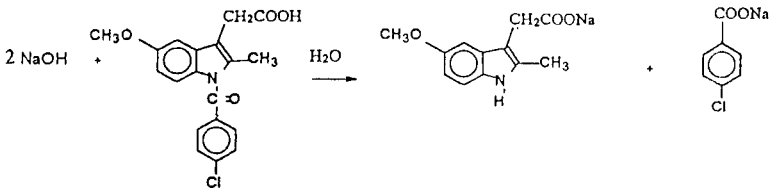
*On leave from Faculty of Pharmacy, University of Costa Rica

INTRODUCTION

The aim of this study was to develop sensitive and selective methods in HPLC to study the pharmacokinetics of indomethacin in rabbit aqueous humour (AH), following the administration of eye drops. The major problem in developing sensitive methods for this biological medium is related to the very small volumes of samples available (ca. 50-100-µl). The performance of electrochemical (EC) and fluorescence (FL) detection were investigated with the objective of improving the limit of quantitation (LOQ) of 10 µg l^{-1} which can be attained in AH, using UV absorption detection (1).

BACKGROUND

Numerous HPLC methods have been proposed in the literature for the determination of indomethacin in biological samples; most of them deal with plasma, serum or urine (2-17), and some with AH (18-19). UV absorption is by far the most commonly used detection mode (1-11,19) with a LOQ which can be as low as 10-20 µg l^{-1} in plasma and about 50-200 µg l^{-1} in urine. In AH, the LOQ is not mentionned by ROVIRA (19), but a 10 µg l^{-1} LOQ is feasible with ACN deproteinisation and UV detection at 266 nm (1). A few authors have used fluorimetric detection after pre (13, 15, 18) or post-column (12, 14, 16) hydrolysis of indomethacin into fluorescent dechlorobenzoylindomethacin (DBI) in plasma and urine (Figure 1). However, when DBI is present as a metabolite in these media, it is necessary to use a difference method with a pre-column reaction (13). In plasma, the best LOQ (and LOQ) for indomethacin is as low as 1 µg l^{-1} (signal-to-noise ratio=2) (15). In urine, the lowest concentration used for calibration was about 250 µg l^{-1} (12, 13). In aqueous humour, the LOD is 50 µg l^{-1} with fluorescence detection after pre-column derivatization (18). The absence of DBI as metabolite in this medium has been mentionned by this author. Electrochemical detection (coulometric detection) has also been used by KAZEMIFARD for indomethacin assay in plasma (17). A LOD of



Indomethacin DBI 4-chlorobenzoic acid, sodium salt

Figure 1. Structure of indomethacin and its hydrolysis products.

20 μg l^{-1} was obtained which was claimed to be at least five-fold lower than UV detection at 254 nm. Since electrochemical and fluorescence detection were shown in a few papers (15, 17) to be significantly more sensitive than UV for the HPLC analysis of indomethacin, both these detection modes were investigated for the routine determination of indomethacin in aqueous humour.

MATERIALS AND METHODS

CHEMICALS

Indomethacin was received as a gift from Chauvin (Montpellier, France).

Methanol from Carlo Erba, Milano, Italy and acetonitrile from Merck, Darmstadt, Germany were of HPLC grade. MilliQ water was used throughout. All other chemicals were of analytical grade.

SOLUTIONS

Stock solutions of indomethacin (500 mg l^{-1} in methanol) for FL and EC detection were prepared and stored up to one month at 4°C. They were suitably diluted in a methanol-water mixture (65:35, v/v) for EC detection and in water for FL detection. These dilutions were stored at 4°C and used within 7 days.

ELECTROCHEMICAL DETECTION

A Hewlett Packard (Walbronn, Germany) Model 1050 isocratic pump and a Rheodyne injector Model 7125 fitted with a 20 µl-loop were used. A UV detector (Hewlett Packard Model 1050) was placed in series with an amperometric detector (Shimadzu L-ECD-6A, Touzart et Matignon, Vitry-sur Seine, France) which was equipped with a glassy carbon working electrode (WE) and a Ag/AgCl reference electrode (RE). The UV detector was set at 266 nm and the detection potential of the WE was set at 1.20V *vs.* Ag/AgCl, unless otherwise stated. This potential was selected from the hydrodynamic voltammogram obtained by successive injections of a standard solution of indomethacin (5 mg l^{-1}) at variable potentials of the WE (from +0.30 V to +1.20 V, *vs.* Ag/ AgCl). Injection was carried out using a Unicam (Cambridge, UK) Model PU 4247 autosampler . The signals were recorded on a dual channel Unicam Model PU 4880 integrator. Separation was carried out on a 125 x 4 mm i. d. Lichrospher (Merck, Darmstadt, Germany) 100 C18 column (5 µm). A disposable guard column (Merck, 4 x 4 mm, i. d.) packed with the same material was used to protect the analytical column. The guard column was replaced after 100 injections. The eluent was a methanol-50 mM sodium phosphate buffer pH 3 (65:35, v/v) mixture, at a flow rate of 1 ml min^{-1}.

FLUORESCENCE DETECTION

Preliminary studies were carried out to follow the formation of DBI together with disappearance of indomethacin in an alkaline medium. For this purpose, a Merck LMC System UV detector set at 266 nm (maximal absorbance wavelength of indomethacin in the mobile phase) was placed in series with a Shimadzu RF-551 fluorescence detector with excitation and emission wavelengths set at 305 and 380 nm, respectively. A Gilson (Villiers-le Bel, France) Model 307 pump fitted with a Rheodyne valve containing a 20-µl loop was used to deliver the mobile phase. The mobile

and stationary phases were the same as those used for EC detection. Signals were recorded on a Shimadzu C-4A dual channel integrator. Off-line derivatization and manual injection were used at this stage. For further experiments and method validation automated derivatization and injection with a Gilson Model 401C dilutor coupled to a Gilson Model 232 XL autosampler with a variable capacity loop were used. The extraction and derivatization procedures were carried out in 800-µl polypropylene minivials. The sample rack for derivatization was themostatted at 40°C ± 0.5°C. The pump and integrator were the same as those described for the preliminary studies.

EXTRACTION PROCEDURE FOR ELECTROCHEMICAL DETECTION

The blank AH matrix used to assess the specificity of EC detection *vs* endogeneous components was treated as follows : an aliquot (200-µl) of drug-free pooled rabbit AH was mixed with an equal volume of acetonitrile. The mixture was vortexed for 15 sec, then centrifuged at 2000 rpm for 10 min. A 20-µl aliquot of supernatant was injected onto the column.

EXTRACTION PROCEDURE FOR FLUORESCENCE DETECTION

To a 50-µl aliquot of AH, 10-µl of 0.1 M acetate buffer pH 5 and 400-µl of ethyl acetate were added. The mixture was vortexed for 2 min, then centrifuged at 2000 rpm for 10 min. The lower aqueous phase was removed by aspiration and discarded. The organic phase was evaporated at 30°C under a stream of nitrogen. The walls of the microvial were washed four times with 50-µl aliquots of ethyl acetate and the combined washings were evaporated again under the same conditions. The microvials were placed in the thermostatted rack of the autosampler for reaction and injection. A 50-µl aliquot of a 50 mM phosphate buffer pH

11.6 was automatically added to each vial and mixed by aspiration with the diluter; the solution was subjected to hydrolysis for 1 hr at 40°C and then 15-µl of a mixture 50 mM phosphate buffer pH 4-ACN (90:10, v/v) was added and mixed. A 50-µl aliquot of this solution was injected onto the chromatographic system.The separation was carried out with a mobile phase 25 mM phosphate buffer pH 6.6-acetonitrile (90:10, v/v) at a flow rate of 1 ml min^{-1}. Peak areas were used for quantitation.

Different criteria for method validation were evaluated. The absence of DBI as metabolite was established by applying the method to four different samples of AH withdrawn 45 min and 2 hr after administration of eye drops to four rabbits. The specificity of the method *vs* the endogeneous components of the matrix was assessed using ten independent sources of AH. Other criteria were assessed on pooled AH. The linearity was assessed on a same day from three calibration curves, each one prepared from matrices spiked between 5 and 50 µg l^{-1} (n= 6 concentrations) of indomethacin. Intra-day precision and accuracy were tested by applying the procedure twelve times on matrices spiked at three concentration levels (5, 20 and 50 µg l^{-1}). Inter-day precision and accuracy were tested on five days from a pool of matrices spiked at the same three concentrations. One aliquot at each concentration was assayed on each of five days with reference to a standard curve daily prepared in the matrix. Analytical recovery was calculated by comparing the peak areas from extracted spiked matrices and non-extracted drug dissolved in the reconstitution solvent at six concentrations (from 5 to 50 µg l^{-1}) of indomethacin on each of five days. Stability studies were carried out on a pool of AH spiked at 5 and 20 µg l^{-1} indomethacin under different conditions: at ambient temperature for 3 hr and 24 hr, after 3 freeze-thaw cycles at -18°C. The stability of the dry residue stored after extraction for 24 hr at -18°C was also tested to cover experimental delays. For each conditions, 6 determinations were carried out by comparison with a calibration curve prepared for each experiment. The results of the assays were compared to those obtained from samples freshly prepared.

RESULTS AND DISCUSSION

ELECTROCHEMICAL DETECTION

Figure 2 shows the plot of the hydrodynamic voltammogram for indomethacin. This compound is oxidised from a potential of +0.70 V (*vs.* Ag/AgCl) with a diffusion plateau observed from a +1.15 V potential. This potential value is higher than that of 0.95 V reported by KAZEMIFARD et al. (17) with a coulometric detector. This difference can be explained by the fact that the coulometric detector has a Pd/H_2 RE which has a higher potential than that of the amperometric detector we used. Figure 3 shows chromatograms recorded at 266 nm (UV) and +1.20V (EC) from drug-free AH and of a standard solution of indomethacin at 50 $\mu g\ l^{-1}$. The LOD in aqueous solutions for EC and UV detection are respectively 20 $\mu g\ l^{-1}$ and 50 $\mu g\ l^{-1}$. The repeatability of successive injections (n=8) of a 5 mg l^{-1} aqueous solution of indomethacin was 0.41% and 1.89% for UV and EC detection, respectively. The lower repeatability in EC detection is related to a progressive decrease in the response which is probably due to adsorption problems at the electrode. This observation together with the low gain in sensitivity shows that EC detection cannot be used routinely for the assay of indomethacin.

FLUORESCENCE DETECTION

PRELIMINARY EXPERIMENTS

Optimisation of the hydrolysis pH and reaction time

Since the hydrolysis of indomethacin is a base catalysed reaction (20-23) a buffer pH 11.6 was selected in our study. A phosphate buffer

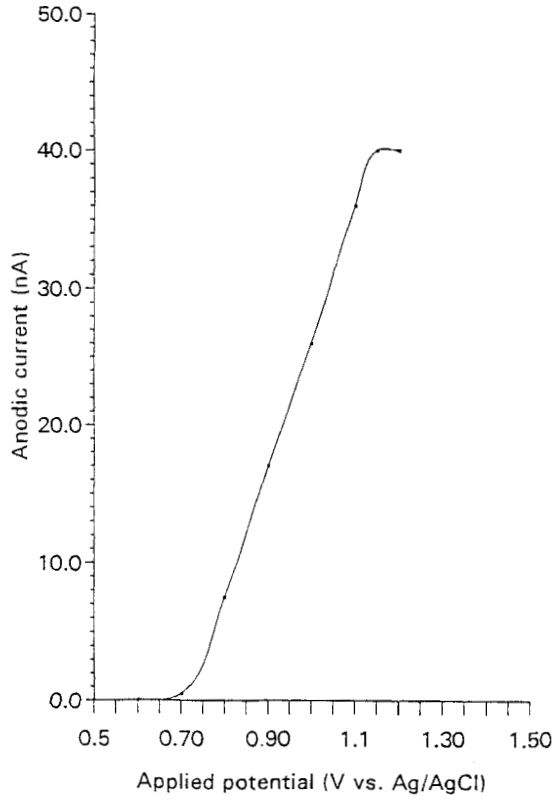


Figure 2. Hydrodynamic voltammogram for indomethacin with amperometric detection. For experimental conditions, see text.

was preferred to a sodium hydroxide solution (15) as it gave us less fluorescent interferences on the chromatogram. The hydrolysis kinetics of indomethacin in a phosphate buffer pH 11.6 (24) was confirmed using both UV and fluorescence detection. Figure 4 shows the formation of the non fluorescent p-chlorobenzoic acid and the fluorescent derivative DBI (13, 20, 25).

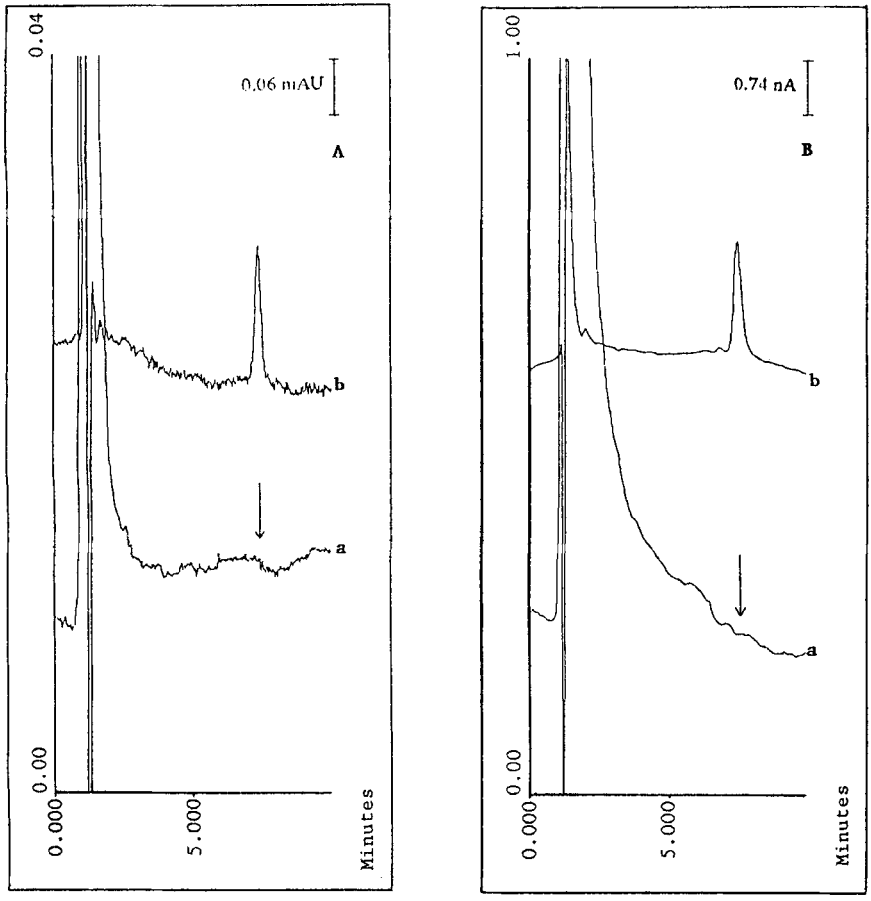


Figure 3. Comparison of UV (A) and electrochemical (B) responses for indomethacin:
(a) blank aqueous humour.
(b) aqueous standard solution (50 $\mu g\ l^{-1}$).
For experimental conditions, see text.

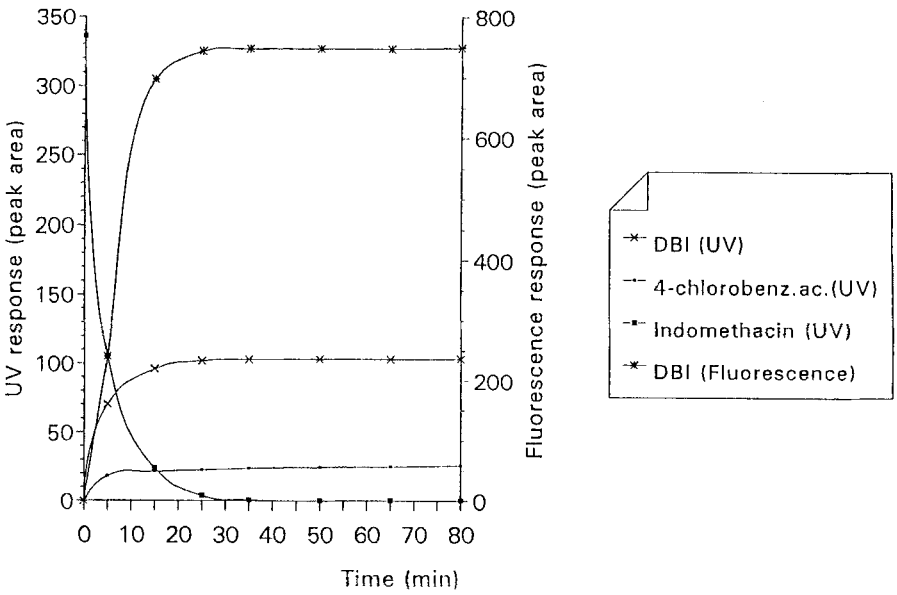


Figure 4. Hydrolysis kinetics of indomethacin followed by UV and fluorescence detection : UV response refers to the left axis (indomethacin, DBI and p-chlorobenzoic acid); fluorescence response (DBI) refers to the right axis. For experimental conditions, see text.

Optimisation of the mobile phase composition

The mobile phase composition was optimized with respect to pH and organic solvent. The influence of pH on the fluorescence response was studied off-line with a spectrofluorimeter, over a pH range compatible with the silica-based C18 column (from pH 3 to 7), keeping a proportion of methanol (65%) identical to that of the mobile phase previously used for chromatography. Increasing the pH resulted in a progressive increase in the fluorescence. A pH of 6.6 gave an increase by 300 % by comparison to a pH of 3. The replacement of methanol by acetonitrile did not produced any significant change in the fluorescence intensity. However, acetonitrile was preferred to methanol for

chromatography as it gave a higher efficiency. A 25 mM phosphate buffer pH 6.6-acetonitrile (90:10, v/v) was finally selected as mobile phase, which gave a typical retention time of 5.15 min (Figure 5) on three different batches of stationary phases.

Optimisation of the extraction procedure

Different solvents proposed in the literature for indomethacin extraction from plasma were used (2,5,9,10,13,15,18). Ethyl acetate with an acetate buffer pH 5 (13,18) was selected as it gave a "clean" blank AH chromatogram and a satisfactory recovery.

Search of DBI as metabolite

Samples withdrawn at different time intervals after administration of indomethacin eye drops to rabbits did not reveal the presence of DBI as metabolite under the operating conditions used. This confirms the observation of SANDERS et al. (18) and previous results of TLC using ^{14}C labelled indomethacin (1).

METHOD VALIDATION

Specificity

Ten individual sources of AH did not give any significant interference at the retention time of indomethacin (Figure 5).

Linearity

The relashionship between the peak areas and concentration of indomethacin was linear between 5 and 50 $\mu g\ l^{-1}$ with a determination coefficient (r^2) of 0.9904.

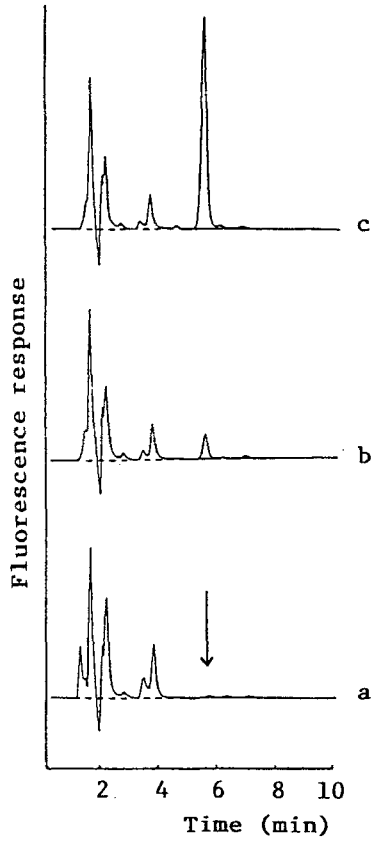


Figure 5. Typical chromatograms using HPLC with fluorescence detection.
(a) blank aqueous humour.
(b) aqueous humour spiked at the LOQ (5 $\mu g\ l^{-1}$).
(c) aqueous humour spiked at 50 $\mu g\ l^{-1}$.

The linear regression equation was :
area = 7943 (± 414) * conc. ($\mu g\ l^{-1}$) - 12507 (± 12561), with the confidence intervals calculated at p=0.05.

The accuracy was evaluated at each concentration by calculating the relative error (%) between the mean calculated value and the actual value. The results are given in Table 1. A concentration of 5 $\mu g\ l^{-1}$ with

TABLE 1. Evaluation of accuracy from linearity data.

Concentration ($\mu g\ l^{-1}$)	Mean bias (%)*
5	18.72
10	-5.53
20	-3.21
30	-0.66
40	-4.91
50	3.83

* n= 3 linearity graphs

TABLE 2. Intra-day and inter-day precision and accuracy

	Intra-day precision and accuracy*		Inter-day precision and accuracy**	
Concentration (µg l-1)	RSD (%)	Bias (%)	RSD (%)	Bias (%)
5	8.05	-1.40	15.15	-1.40
20	7.25	-9.95	5.27	7.25
50	4.89	-2.14	12.03	0.02

* 12 assays at each concentration, on a same day

** 1 assay at each concentration on each of 5 days

a relative error lower than 20% complies with the guidelines for accuracy of the Washington Conference (26) with respect to the LOQ. This concentration was further tested for both accuracy and precision using samples spiked at this level, independently from the calibration curve and from runs performed on different days.

Precision and accuracy

The results of intra-day (12 determinations per day) and inter-day (1 assay on each of 5 days) precision and accuracy at three concentration levels are given in Table 2. A two-way ANOVA showed that there was no day influence on the inter-day results.

TABLE 3. Analytical recovery of indomethacin from aqueous humour (n=5 days).

Concentration (µg l-1)	Mean Recovery (%)	RSD (%)
5	65.16	9.32
10	75.14	10.97
20	67.63	2.35
30	67.56	12.05
40	72.48	3.92
50	71.24	4.98
Mean	69.87	5.31

LOQ and LOD

Based on accuracy and precision results (lower than 20 %), a concentration of 5 µg l^{-1} can be considered as the LOQ (26) in AH. The LOD was determined to be 2 µg l^{-1} (S/N =3) and confirmed by analyzing ten different matrices spiked at this concentration.

Analytical recovery

Table 3 gives the results of recoveries over the concentration range studied. The mean recovery is approximately 70% with a RSD of 5.31%.

Stability of indomethacin in AH

The confidence intervals calculated on the results showed that the results were not affected by storage at 24 hr at ambient temperature or three freeze-thaw cycles. Moreover, dry extracts can be kept at 18°C for 12 hr should this be necessary.

CONCLUSION

The present study showed that ECD coupled with HPLC does not present any advantage over UV detection for indomethacin monitoring

in AH. However, liquid-liquid extraction of indomethacin from this matrix followed by automated pre-column hydrolysis and fluorescence detection yields a LOD and a LOQ lowered by a factor of two compared with to the UV detection method presently in use (1). Validation results with fluorescence detection showed that the proposed procedure complies with the validation guidelines for biological assays (26). It is well suited to the determination of indomethacin in AH, as the fluorescent derivative formed (DBI) is not present as metabolite in this matrix. However, this detection mode should be used only when a high sensitivity is required, as it is more time consuming and difficult to implement when compared with the HPLC-UV technique.

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