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Determination of sulindac and its metabolites in human serum by reversed-phase high-performance liquid chromatography using on-line post-column ultraviolet irradiation and fluorescence detection

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Abstract

On irradiation with ultraviolet light, the antiinflammatory agent sulindac and its two metabolites sulindac sulfone and sulindac sulfide form highly fluorescent derivatives. This reaction was exploited for the sensitive and selective detection of these compounds in serum using reversed-phase high-performance liquid chromatography on a Ultrasphere octylsilane column (150 × 4.6 mm I.D.) at ambient temperature with a flow-rate of 0.5 ml/min. The analytes of interest were isolated from serum using a Bond-Elut C_2 column with satisfactory recovery and selectivity. The detection limits were 10 ng/ml for each of the three analytes using 1 ml of serum and the limit of quantitation was 50 ng/ml. Linear calibration curves from 50 to 1000 ng/ml for all three analytes show coefficients of determination of 0.9999. The post-column ultraviolet irradiation was optimized and the effect of irradiation time on the fluorescence response was determined for all three analytes. Precision and accuracy of the method were 0.4–5.6 and 1.6–4.5% for sulindac, 2.3–5.6 and 1.4–5.3% for sulindac sulfone and 2.5–4.3 and 0.8–2.8% for sulindac sulfide, respectively.

1. Introduction

On-line post-column UV irradiation in HPLC has been increasingly used in the recent past to convert non-fluorescent or poorly fluorescent analytes to highly fluorescent species [1–8]. The photochemical reactor is easy to construct and a commercial version is also available. A number of reviews have been published on post-column photochemical detection in HPLC [9–11].

Sulindac is a non-steroidal anti-inflammatory agent with analgesic and antipyretic properties. It is used in the long-term treatment of various arthritic conditions [12,13]. It causes significantly less gastrointestinal irritation and fecal blood loss than therapeutically equivalent doses of aspirin [14]. The technique which is commonly used for the analysis of sulindac and its metabolites is HPLC with UV detection. All previously reported literature methods use solvent programming to reduce the assay time and liquid–liquid extraction, which involves tedious and time-consuming evaporation steps for the separation of sulindac and its metabolites from endogenous

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substances [15–17]. Existing procedures have run times upto 15 min using gradient programming.

This paper describes an isocratic HPLC method using post-column UV irradiation and solid-phase extraction (SPE) to measure ng/ml concentrations of sulindac and its sulfone and sulfide metabolites in serum and obviates the need for costly equipment such as solvent programming to give good sensitivity, selectivity and short chromatographic run time. The method is linear upto 1000 ng/ml sulindac and its two metabolites. The assay procedure possesses the necessary sensitivity to be useful for clinical studies of sulindac doses of 100–200 mg.

2. Experimental

2.1. Reagents

Sulindac and indomethacin (internal standard) were obtained from Sigma (St. Louis, MO, USA). Sulindac sulfone and sulindac sulfide were donated by Merck (Rahway, NJ, USA). Blank human serum (Cat. No. 2906-34) was obtained from Fisher Scientific (Orangeburg, NY, USA). The solvents used were all HPLC grade. Acetonitrile, absolute methanol, concentrated phosphoric acid and potassium dihydrogenphosphate were obtained from J.T. Baker (Phillipsburg, NJ, USA). All chromatographic solutions were filtered through a 0.45-μm filter (Alltech Assoc., Deerfield, IL, USA).

2.2. Preparation of stock and standard solutions

Individual stock solutions of sulindac, its sulfone and sulfide metabolites and the internal standard indomethacin were prepared in absolute methanol to give concentrations of 100 μg/ml. Appropriate volumes of the three analytes and the internal standard were pipetted into a 1-ml volumetric flask and serum added to volume to give final serum concentrations of 50, 250 and 1000 ng/ml of each analyte and 7.5 μg/ml of the internal standard.

2.3. HPLC system

A Model 110B HPLC pump (Beckman, Fullerton, CA, USA) and a Model 728 autosampler (Micromeritics, Norcross, GA, USA) equipped with a 100-μl loop were used for the analysis. The stationary phase was a octylsilane column (Ultrasphere C_8, 5 μm, 150 mm $\times$ 4.6 mm I.D., Beckman). The mobile phase consisted of 0.068 *M* phosphate buffer (pH 2.5)–acetonitrile (45:55, v/v). The flow-rate was 0.5 ml/min and the column was at ambient temperature (22 $\pm$ 1°C). A Model 1046A programmable fluorescence detector (Hewlett Packard, Avondale, PA, USA) was used with an excitation wavelength of 232 nm and an emission filter of 335 nm. A HP 3395 integrator (Hewlett Packard) was used to record each chromatogram and peak-height responses.

2.4. Post-column photochemical reactor

The photochemical reactor was equipped with an SC3-9 UV lamp and SCT-4 power supply (UVP, San Gabriel, CA, USA). The irradiation coil (790 cm $\times$ 0.3 mm I.D.) was made from PTFE tubing (Anspec, Ann Arbor, MI, USA). The reaction coil was knitted in a manner similar to that described in the literature, but with minor modifications [18]. In contrast to the reported method, where the knitter used a 3- or 4-peg "Strickliesel" which resulted in a dense rope-like configuration, the coil used in this experiment was knitted around 6 pegs which resulted in a hollow cylinder. The coil was snug onto the surface of the lamp in a sleeve-like manner. A 3 in. (ca. 7.6 cm) diameter fan (Rotron, Woodstock, NY, USA) was provided for air cooling. A box (31.5 $\times$ 16.5 $\times$ 15.5 cm) constructed from galvanized sheet steel and perforated masonite was used to house the UV lamp, irradiation coil and fan.

2.5. Assay procedure

One drop of saturated ammonium sulphate solution and one drop of 1 *M* HCl were added to 1-ml serum samples spiked with appropriate concentrations of the three analytes to give final

concentrations of 100 and 500 ng/ml. The samples were vortex-mixed for 3 min and then passed through a C_2 Bond-Elut SPE column attached to a vacuum manifold (Vac-Elut, Varian, Harbor City, CA, USA). The column was previously conditioned with 1 ml of methanol followed by 1 ml of mobile phase. The column was washed with 6×500 μl of acidified water (pH 3.0, adjusted with phosphoric acid)–acetonitrile (8:2, v/v) and allowed to dry for 3 min. The analytes of interest were eluted with 4×250 μl of mobile phase and the tubes were vortex-mixed to ensure homogeneity prior to analysis by HPLC. A 100-μl aliquot was injected into the HPLC system.

For absolute recovery experiments, spiked samples were compared to unextracted stock solutions. Drug peak-height ratios were used to calculate the recoveries.

2.6. Preparation of standard calibration curve

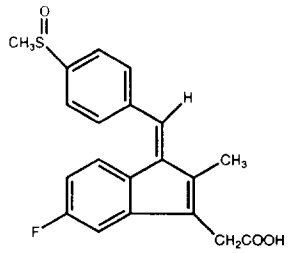
Linear calibration curves were constructed in the range 50–1000 ng/ml using 50, 250 and 1000 ng/ml concentrations of each analyte.

3. Results and discussion

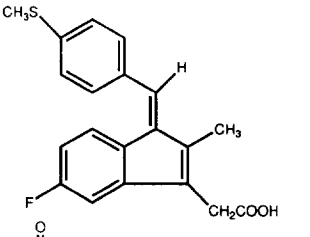
The chemical structures of sulindac, the sulfone and sulfide metabolites and indomethacin (internal standard) are shown in Fig. 1. In the course of this investigation of photochemical reaction–fluorescence detection for the HPLC analysis of various drugs and their metabolites, sulindac and its metabolites were found to have no native fluorescence, but produced fluorescent species upon UV irradiation. The assay of sulindac and metabolites could also benefit from a rapid and less tedious sample preparation method and simple isocratic elution without any solvent programming steps.

3.1. Post-column photochemical derivatization

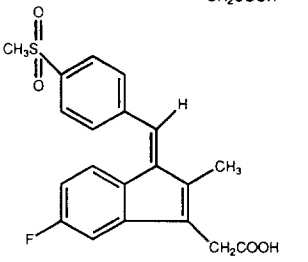
The effect of irradiation time on the fluorescence response was determined by recording the responses of all three analytes at various flow-



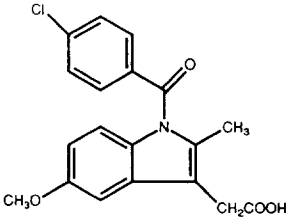
Sulindac



Sulindac sulfide



Sulindac sulfone



Indomethacin

Fig. 1. Structures of the analytes and the internal standard.

rates (0.2–1.0 ml/min) which corresponded to an irradiation time of 2.8 min to 33.4 s with an irradiation coil length of 790 cm. From Fig. 2 it can be seen that the irradiation time had a profound effect on the fluorescence response for all three analytes. Although the fluorescence response was maximum at 0.3 ml/min, it was not used for the chromatography due to excessive band broadening (Tailing factor (T_f) of 1.5, 1.4 and 1.5 for sulindac, the sulfone and the sulfide, respectively). A slightly higher flow-rate of 0.5 ml/min was selected for the assay method since chromatography gave more acceptable peak

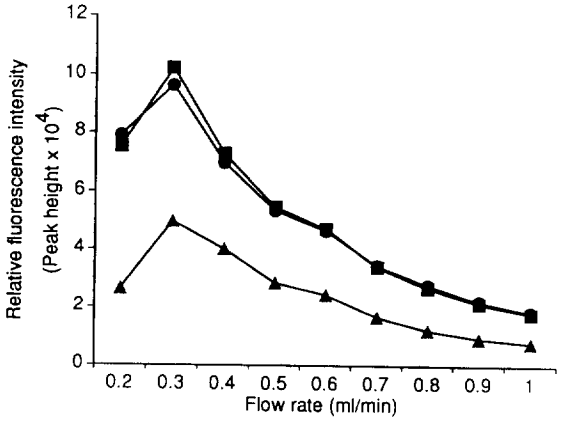


Fig. 2. Peak-height response versus flow-rate for sulindac (■), sulindac sulfone (●) and sulindac sulfide (▲) after post-column UV irradiation and fluorescence detection. The concentrations used are 500 ng/ml for sulindac and sulfone metabolite and 225 ng/ml for sulfide metabolite.

shapes (T_f of 1.1, 1.2 and 1.3 for sulindac, its sulfone and sulfide, respectively) without a significant loss of sensitivity.

Fig. 3A shows the chromatogram of a serum blank with the UV lamp on and Fig. 3B shows the chromatogram of the analytes and internal standard with the UV lamp on. With the UV lamp off, all four compounds gave no detectable response and the chromatogram is similar to the blank with the lamp on. The use of an octylsilane reversed-phase column enabled the analytes to be separated within a reasonable chromatographic run time under isocratic conditions.

To decrease the sample manipulation and preparation time involved in liquid–liquid extraction by previous reported methods, a simple SPE procedure was developed for sample cleanup. The C_2 sorbent was selected because it allowed an excellent elution of sulindac and its metabolites using the HPLC mobile phase as eluent. Absolute recoveries of >90% were obtained for all three analytes. SPE experiments using the more lipophilic C_8 and C_{18} SPE columns gave less than 80 and 65% recoveries,

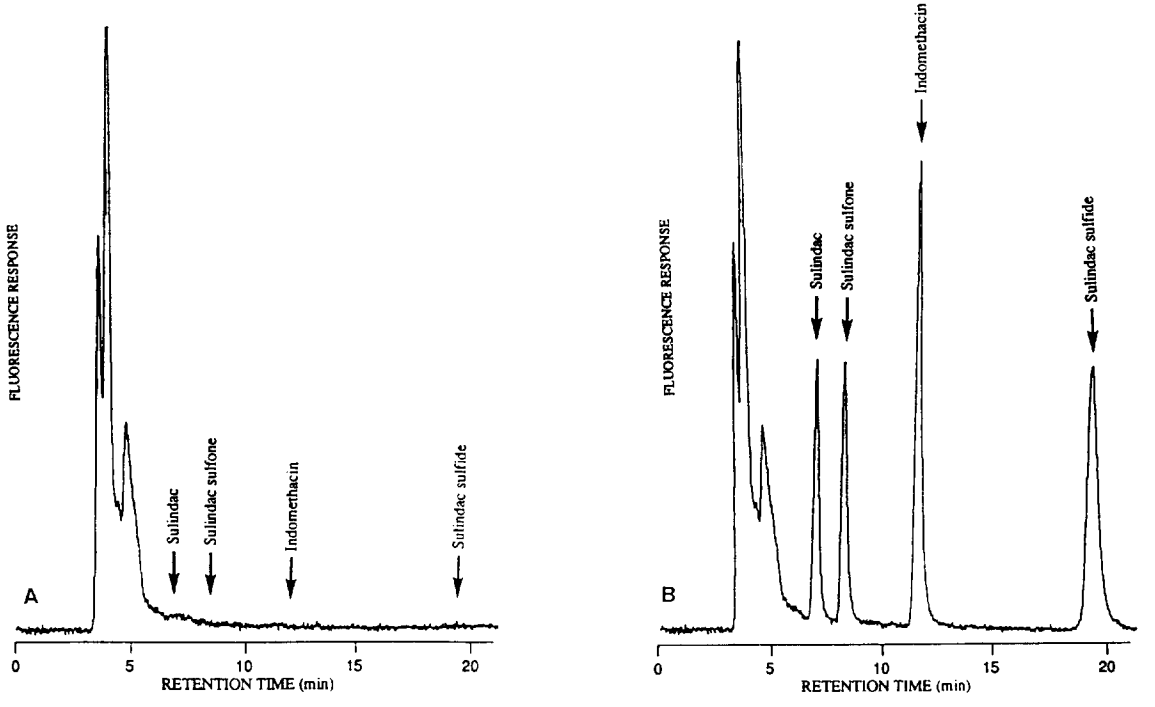


Fig. 3. (A) Representative chromatogram of serum blank with UV lamp on. (B) Representative chromatogram of sulindac (100 ng/ml), sulindac sulfone (100 ng/ml), indomethacin (7.5 μg/ml, internal standard) and sulindac sulfide (100 ng/ml) with UV lamp on.

respectively, using 1 ml of mobile phase as eluent. The mean absolute recoveries using the C_2 SPE were 92.3 ± 2.3% for sulindac, 91.8 ± 3.3% for sulindac sulfone and 90.4 ± 2.4% for sulindac sulfide ($n = 3$). Indomethacin was selected as the internal standard because it also produced a highly fluorescent species on UV irradiation and behaved similarly through the sample preparation processes with good recovery (88 ± 4.1%).

The limit of detection, based on a signal-to-noise ratio of 5, was determined to be 10 ng/ml for sulindac and its metabolites ($n = 3$).

The calibration curves showed good linearity in the 50–1000 ng/ml range for all three analytes. The coefficients of determination were more than 0.9999 for sulindac and the sulfone and sulfide metabolites. Representative linear regression equations obtained for sulindac, sulindac sulfone and sulindac sulfide were $y = 0.005924x + 0.029202$, $y = 0.009804x + 0.011461$ and $y = 0.007885x + 0.013408$, respectively, where y and x are the drug-to-internal standard peak-height ratios and concentration of the analytes, respectively. The intra-day precision and accuracy ($n = 3$) as expressed by %R.S.D. and %error were 2.4–5.6% and 2.4–4.5% for sulindac, 5.4–5.6% and 1.4–5.3% for sulindac sulfone and 3.3–4.3% and 2.4–2.8% for sulindac sulfide, respectively. The inter-day precision and accuracy ($n = 9$, for three days) were 0.4–2.7% and 1.6–2.1% for sulindac, 2.3–3.0% and 2.0–3.5% for sulindac sulfone and 2.5–3.2% and 0.8–1.3% for sulindac sulfide, respectively. The detailed data is listed in Table 1. The intra-day precision and accuracy ($n = 3$) at the 50 ng/ml level were 6.8% and 5.2% for sulindac, 4.3% and 4.4% for sulindac sulfone and 4.4% and 0.6% for sulindac sulfide, respectively. The inter-day precision and accuracy ($n = 9$) at 50 ng/ml were 5.1% and 3.8% for sulindac, 1.5% and 3.8% for sulindac sulfone and 3.8% and 4.7% for sulindac sulfide, respectively.

In summary, a precise, accurate and rapid isocratic HPLC method employing SPE and post-column UV irradiation has been developed for the analysis of sulindac and its sulfone and sulfide metabolites in human serum. The method is sensitive to 10 ng/ml of the three analytes in human serum. The total run time of the isocratic method was less than 21 min and is a considerable improvement over existing HPLC methods.

Table 1
Accuracy and precision of human serum samples with added sulindac, sulindac sulfone and sulindac sulfide

	Concentration added (ng/ml)	Concentration found (ng/l)[a,b]	Error (%)	R.S.D. (%)
Intra-day				
Sulindac	100	95.51 ± 2.23	4.5	2.4
	500	488.31 ± 27.21	2.4	5.6
Sulindac sulfone	100	94.71 ± 5.1	5.3	5.4
	500	506.79 ± 28.34	1.4	5.6
Sulindac sulfide	100	97.19 ± 4.15	2.8	4.3
	500	511.94 + 16.79	2.4	3.3
Inter-day				
Sulindac	100	98.41 ± 2.65	1.6	2.7
	500	489.61 ± 1.88	2.1	0.4
Sulindac sulfone	100	96.50 ± 2.2	3.5	2.3
	500	510.15 ± 15.2	2.0	3.0
Sulindac sulfide	100	99.27 ± 2.51	0.8	2.5
	500	506.14 ± 16.37	1.3	3.2

[a] Based on $n = 3$, for intra-day assay.
[b] Based on $n = 9$, for inter-day assay.

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