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Note

Determination of 6,11-dihydro-11-dibenz[*b*,*e*]oxepin-2-acetic acid (isoxepac) in plasma by high-performance liquid chromatography

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6,11-Dihydro-11-oxodibenz[*b*,*e*]oxepin-2-acetic acid (HP 549, isoxepac, Hoechst, Frankfurt/M, G.F.R.) is an anti-inflammatory and analgesic agent presently undergoing extensive clinical trials in man. In our department the possibility of pharmacokinetic interaction between acetylsalicylic acid (aspirin) and isoxepac during acute and long-term therapy of both agents, is being investigated. The gas—liquid chromatographic method described by Bryce and Burrows [1] was used by us initially, but found to be too time consuming. The large number of samples which had to be analysed for salicylic acid and isoxepac required the development of an analytical method for isoxepac which would be sensitive, accurate, precise and simple from the point of view of time consumption. This paper describes a high-performance liquid chromatographic (HPLC) method, using native fluorescence detection, for the determination of isoxepac in plasma. The assay is sufficiently sensitive to follow reliably plasma levels of isoxepac for a period of five half-lives after a therapeutic dose of 100 mg.

EXPERIMENTAL

Reagents

The chemical structures of isoxepac and the internal standard, 6,11-dihydro-11-oxodibenz[*b*,*e*]oxepin-3-propionic acid are shown in Fig. 1. Both compounds were obtained from Hoechst.

Methanol, methylene chloride and acetic acid were guaranteed reagent grade (Merck, Darmstadt, G.F.R.) and were used as received.

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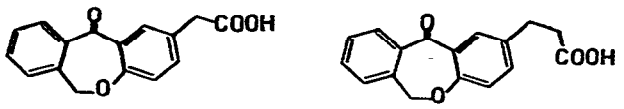

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Fig. 1. Chemical structures of isoxepac (1) and 6,11-dihydro-11-oxodibenz[*b,e*]oxepin-3-propionic acid (2).

Apparatus

An M6000A pump and a WISP autosampler (Waters Assoc., Milford, MA, U.S.A.) were coupled to a Radial-Pak C_{18} (10 μm) cartridge (10 cm × 8 mm high-density polyethylene column packed with octadecylsilane-bonded porous silica) held in an RCM-100 Radial compression unit (Waters Assoc.). A Fluorichrome fluorescence detector (Varian, Walnut Creek, CA, U.S.A.) was used to measure fluorescence of the eluate. Alternatively, a Model 450 variable-wavelength detector (Waters Assoc.) was used. The results were processed on a Waters 730 data module in the peak-height mode.

Other apparatus used consisted of glass centrifuge tubes with B19 ground glass joints and stoppers, a variable-speed multi-purpose rotator (Scientific Instruments, Springfield, MA, U.S.A.), a Clements Model B universal centrifuge, (H.I. Clements, Sydney, Australia), 1-, 2- and 5-ml glass ampoules, high-purity nitrogen and a 100-μl Hamilton syringe.

Stock solutions

A stock solution of the internal standard was prepared by dissolving 2.6 mg of the internal standard in 10 ml 0.02 *M* sodium carbonate solution. Aliquots (0.5 ml) of stock solution were kept frozen (—20°C) in sealed glass ampoules.

Plasma standards

An accurately weighed amount of isoxepac was dissolved in a weighed amount of fresh human plasma by shaking for 4 h. By using an average density of 1.027 for plasma [2] isoxepac concentration can be calculated. Weighed amounts of this plasma stock solution were further diluted with weighed amounts of plasma to obtain standards with lower concentrations of isoxepac. Aliquots (1.5 ml) of these standard plasmas were stored frozen (—20°C) in 2-ml sealed glass ampoules.

Extraction

To 1 ml plasma (standard or unknown) in a 10-ml B19 ground glass centrifuge tube was added 20 μl internal standard solution followed by 0.25 ml 1 *M* hydrochloric acid and 5 ml methylene chloride. The stoppered tubes were rotated for 5 min at a speed of 10 rpm on a rotator and were then centrifuged at 900 *g* for 10 min at room temperature. Emulsions which formed very easily during the extraction procedure were found to be broken more readily by centrifuging at room temperature than by centrifuging in a refrigerated centrifuge. The supernatant aqueous layer was aspirated off and the organic phase transferred to 5-ml glass ampoules in which the solvent was evaporated at 40°C under a gentle stream of high-purity nitrogen.

The residue in the ampoules was dissolved in 100 μl of the mobile phase used for chromatography and 60 μl of this solution were injected for analysis.

Chromatography

The mobile phase consisted of methanol—double distilled water—glacial acetic acid (550:450:2). A constant flow-rate of 2 ml/min was maintained with a pressure of about 60 bar at ambient temperature (24°C) through a Radial-Pak C18 (10 μm) column. Excitation energy was obtained through glass band filters 7-54 with 7-60 giving an excitation band maximum at 340—380 nm while emission energy was monitored above 400 nm by using glass cutoff emission filters 3-73 which cut off energy below 400 nm, combined with a wide-band filter 4-76 which prevents transmission of long-wavelength red leakage above 600 nm.

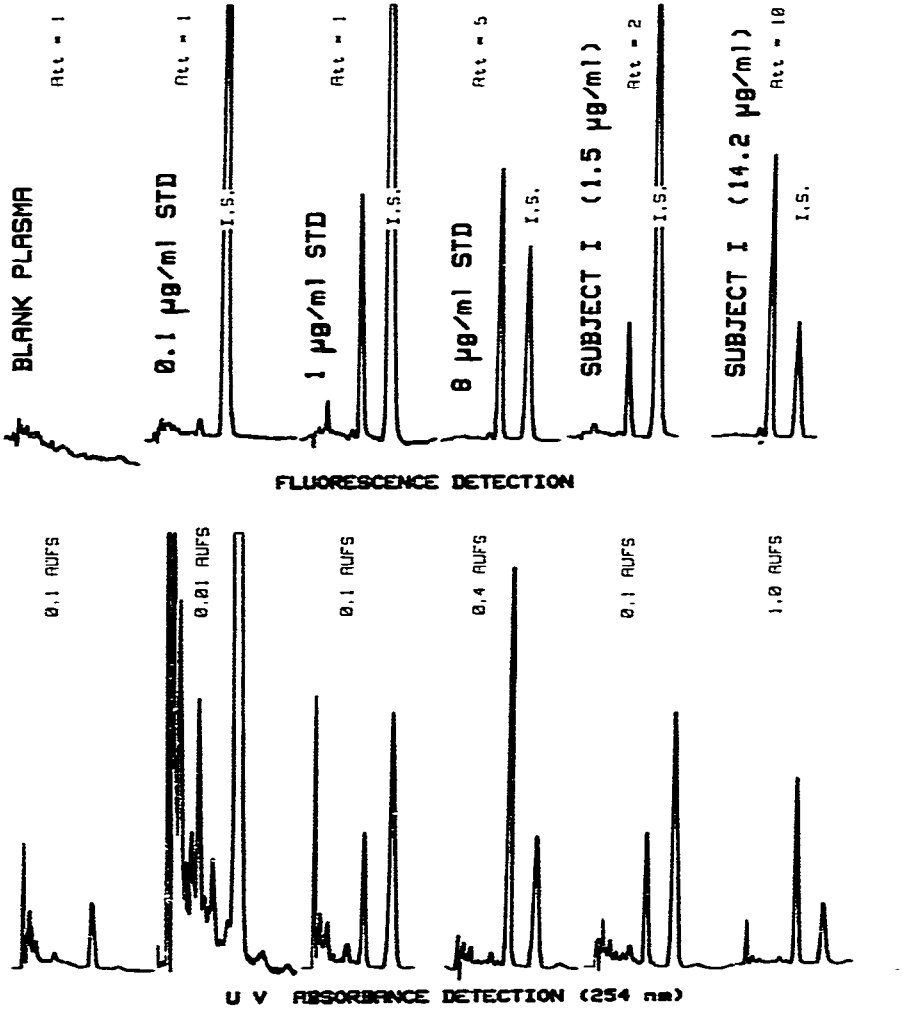

Fig. 2. Typical chromatograms of plasma samples.

Retention times of isoxepac and the internal standard were approximately 7 and 11 min respectively.

RESULTS AND DISCUSSION

Fig. 2 shows representative chromatograms obtained and demonstrates the lack of interfering endogenous compounds.

For comparison representative chromatograms obtained when monitoring absorbance of the same samples at 254 nm instead of fluorescence are shown. Although good results were obtained an interfering UV-absorbing peak with retention time very similar to the internal standard favours the use of the fluorescence detector.

Linear calibration curves of isoxepac peak height/internal standard peak height versus plasma concentrations were obtained with plasma standards containing 2—32 μg/ml. The lines passed close to the origin and the slopes remained relatively constant as can be seen from the following equation which represents the average of twelve calibration curves (obtained by linear regression analysis) constructed during a period of six weeks while the assays of isoxepac in actual plasma samples of the interaction study were being carried out: $y = (9.179 \pm 0.602)x - (0.262 \pm 0.342)$. Correlation coefficients for these linear regressions were consistently greater than 0.998 ($n = 4$) making one-point calibration feasible.

A summary of the results with spiked plasma samples using the fluorescence detector during the validation period of this assay method and with samples processed as quality controls during the period of the interaction study over a period of six weeks is presented in Table I. For comparison, results obtained

TABLE I

RECOVERY OF ISOXEPAC IN SPIKED PLASMA SAMPLES

Concentration spiked (μg/ml)		Mean concentration found (μg/ml)	C.V. (%)	*N*
a*	39.07	37.65	3.1	4
	30.00	32.13	4.3	3
	14.86	14.83	4.9	4
	5.29	5.08	3.0	4
	1.20	1.26	4.5	4
	0.60	0.64	7.5	4
	0.12	0.14	6.5	4
b**	22.11	21.05	1.4	4
	14.34	13.53	8.6	4
	7.16	7.16	0.6	4
	2.77	2.83	0.9	4
c***	29.01	27.25	6.1	11
	17.41	17.68	10.5	11
	5.80	5.73	5.1	9
	2.90	2.97	9.4	9

*(a) Fluorescence detection during validation period.
**(b) UV absorbance detection (254 nm) during validation period.
***(c) Fluorescence detection: quality controls during clinical trial.

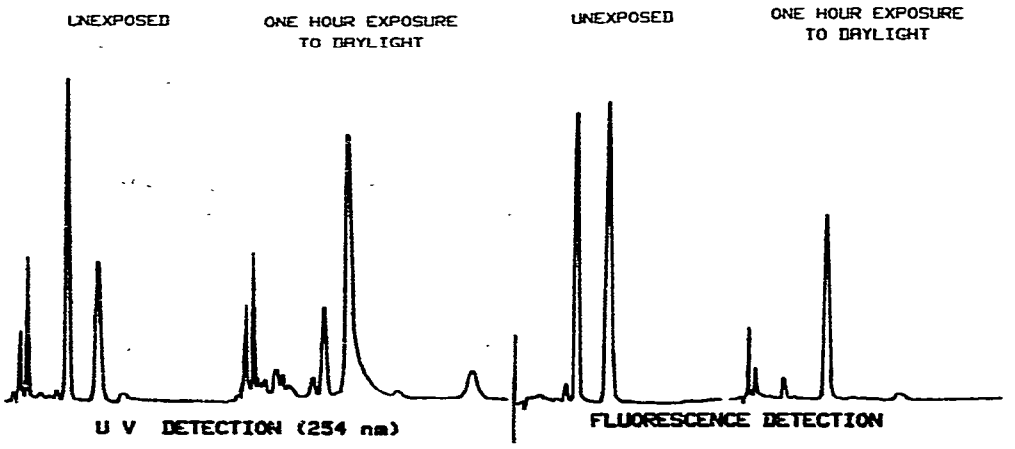

Fig. 3. Effect of exposing plasma extracts, dissolved in mobile phase, to daylight.

with the UV absorbance detector during the validation period are also included in Table I. Although excellent results were obtained with UV (254 nm) detection it was felt that the accuracy and precision could be adversely influenced by the component in normal plasma which eluted with almost the same retention time as the internal standard. The good accuracy and precision obtained during the validation period could be ascribed to the fact that the spiked plasma samples and plasma standards were made up from a homogeneous plasma pool.

The extraction procedure with methylene chloride is that described by Bryce and Burrows [1] and no other solvents were evaluated. The absolute extraction yield of isoxepac carried through the extraction procedure with spiked plasma samples containing 10 μg/ml isoxepac and using 6,11-dihydro-11-oxodibenz-[*b*,*e*]oxepin-3-propionic acid as an external standard was 82%. That of the internal standard at 10 μg/ml, using isoxepac as external standard, was 70%.

The stipulation by Bryce and Burrows [1] that all operations be carried out in subdued light was found to be very important. Fig. 3 shows chromatograms of extracts which, after dissolution in mobile phase, were injected after minimum exposure to strong daylight conditions compared with the same extracts after exposure of the dissolved extract for 1 h to daylight prevailing on a window sill in the laboratory. This effect was not observed with dissolved extracts kept in the dark for several hours.

Specificity

Since this method was used only during a study of the possible pharmacokinetic interaction of isoxepac with acetylsalicylic acid, only salicylic acid was tested for interference with this assay. No interference by salicylic acid was observed up to concentrations of 400 μg/ml, in fact, salicylic acid can be quantitated simultaneously with isoxepac in the same plasma samples provided care is taken to prevent sublimation of the salicylic acid during the solvent evaporation stage.

REFERENCES

1 T.A. Bryce and J.L. Burrows, J. Chromatogr., 145 (1978) 393.
2 Documenta Geigy, 6th ed., J.E. Geigy, Basle, 1962.