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LIQUID CHROMATOGRAPHIC DETERMINATION OF INDOLIDAN AND ITS DEHYDRO METABOLITE IN PLASMA

USE OF THE VARIAN AASP® FOR SOLID-PHASE EXTRACTION

MARTA HAMILTON

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285 (U.S.A.)

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SUMMARY

A method is described for the determination of indolidan and its dehydro metabolite in human plasma. Two procedures are given, one manual and one which is semi-automated by use of the Varian AASP® (Automated Analytical Sample Processor). The method involves solid-phase extraction to isolate the drug, metabolite, and an internal standard from plasma. The manual procedure requires elution of the analytes from the extraction column with methanol, evaporation of the methanol to dryness, and reconstitution in mobile phase prior to injection into a reversed-phase liquid chromatographic system. Using the AASP® (semi-automated procedure), the analytes are eluted from the extraction column directly into the chromatographic system, resulting in substantial savings in sample preparation time. The limit of detection for both drug and metabolite is 0.25 ng/ml in plasma. Detection was made by ultraviolet absorbance, using wavelength programming to optimize the sensitivity of the assay for each compound.

INTRODUCTION

Indolidan (LY195115), 1,3-dihydro-3,3-dimethyl-5-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2H-indol-2-one (I, Fig. 1), is a new (+) inotrope currently under development for the treatment of congestive heart failure. Its primary metabolite in man is dehydroindolidan (II, Fig. 1) which possesses approximately equal in vitro pharmacological activity [1].

The development of this potent compound presented a particular challenge to our analytical laboratory. A method was needed to determine the concentrations of the drug and metabolite in human plasma with a detection limit of less than 0.5 ng/ml. To this end, a liquid chromatographic procedure has been developed which utilizes recent advances in sample preparation and detector technology.

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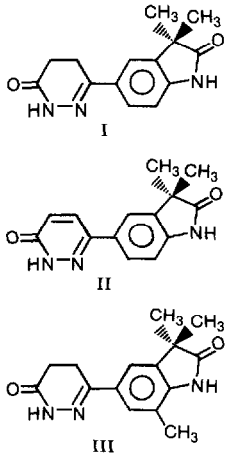

Fig. 1. Structures of indolidan (I), its dehydro metabolite (II), and the internal standard (III).

The method, presented here in both manual and semi-automated modes, is sensitive to 0.25 ng/ml each of drug and metabolite in human plasma.

Solid-phase extraction columns have become increasingly popular due to the selectivity offered by the numerous types of phases available and the opportunity for solvent exchange. In this procedure, the drug, metabolite, and an internal standard are retained on an extraction column and either eluted with solvent for subsequent dry down and reconstitution in mobile phase (manual procedure) or processed in a semi-automated mode by use of the Varian AASP® (Automated Analytical Sampler Processor). The AASP utilizes cassettes of ten solid-phase extraction cartridges, which, following sample appliction, are placed in the loading tray of the device. Each cartridge on the cassette is encapsulated in turn and placed in-line between the chromatographic system pump and the analytical column. The analytes are thus eluted from the extraction cartridge directly into the chromatographic system, obviating the need for eluent collection and evaporation and allowing the total sample extract to be analyzed. The statistical results for the method were similar for the two modes of operation, however, the AASP proved to be a substantial time saver. The sample preparation time was essentially halved.

The detection of the drug and metabolite was optimized through the use of a programmable-wavelength UV detector. This allowed for the monitoring of each analyte at the wavelength of optimal absorbance.

EXPERIMENTAL

Chemicals and reagents

Indolidan, its dehydro metabolite, and the internal standard, 1,3-dihydro-3,3,7-trimethyl-5-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2H-indol-2-one (III, Fig. 1), were obtained from Eli Lilly and Company (Indianapolis, IN, U.S.A.). C_2 solid phase extraction columns (Bond-Elut®, 1 ml, 100 mg size), C_2 solid phase

extraction cassettes, and the sample processing station for the extraction columns (Vac-Elut®) were purchased from Analytichem International (Harbor City, CA, U.S.A.). The processing station for the cassettes was purchased from Varian Assoc. (Walnut Creek, CA, U.S.A.). Methanol, hexane and tetrahydrofuran (distilled-in-glass) were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Distilled, deionized water was used in all procedures. All other chemicals were of analytical reagent grade.

Liquid chromatography

The liquid chromatograph consisted of an isocratic pump (Beckman Model 114, Sam Ramon, CA, U.S.A.), connected to a pulse dampener (SSI, Alltech Assoc., Deerfield, IL, U.S.A.), a programmable UV detector (Kratos Model 783, Applied Biosystems, Foster City, CA, U.S.A.), and either an autosampler (WISP® Model 710B, Waters Assoc., Milford, CT, U.S.A.) or the AASP automated sample processor (Varian Assoc.). The analytical column was a 25 cm×4.6 mm I.D. Zorbax phenyl, 5 μm (DuPont, Wilmington, DE, U.S.A.). A guard column (direct-connect, Alltech Assoc.) was repacked daily with pellicular packing (Co:Pell® ODS, Whatman, Clifton, NJ, U.S.A.). An in-line filter (0.2 μm, Upchurch Scientific, Oak Harbor, WI, U.S.A.) was positioned between the injector and the pre-column. On-line data acquisition and subsequent calculations were performed by a Hewlett-Packard Model 1000 computer.

The mobile phase was prepared by mixing 860 ml of 0.01 *M* ammonium acetate, pH 5.5 (filtered through a 0.2-μm Nylon-66 membrane), with 140 ml of tetrahydrofuran. The mixture was degassed gently before use. The flow-rate was 1.0 ml/min and the column temperature ambient. The detector was programmed such that indolidan and the internal standard were detected at 310 nm and the dehydro metabolite at 280 nm. Autozeros were programmed to occur prior to the elution of indolidan and immediately following each wavelength change. The detector range was set at 0.002 a.u.f.s.

Standard solutions

Standards in plasma were prepared to contain 0.5, 1.0, 2.5, 5.0, and 10.0 ng/ml each of indolidan and its dehydro metabolite. The internal standard solution was prepared by dissolving 1 mg in 100 ml of methanol. Subsequent dilutions of this solution were made in water to give a final concentration of 40 ng/ml.

Sample preparation procedures

Aliquots of plasma samples or standards (1 ml) were dispensed into disposable glass tubes with PTFE-lined screw caps, and 100 μl of the internal standard solution were added to each. The tubes were placed in a refrigerator for approximately 5 min. Hexane (3 ml) was then added and the tubes were rotated gently for 5 min. Following centrifugation, the hexane layer was aspirated and discarded. The aqueous layer was further prepared by one of the following procedures.

Manual procedure. The solid-phase extraction columns were attached to plastic reservoirs by the use of adaptors and positioned on the vacuum box. The columns were conditioned with 2 ml of methanol followed by 2 ml of water. This was

accomplished by placing the solvents in each reservoir and using a vacuum applied to the box to pull them through the extraction columns. A small volume of water was left in each column to prevent the packing from drying before the samples could be applied. Using disposable pipets, the hexane-washed samples were transferred to the reservoirs and pulled by vacuum onto the extraction columns. Each tube was rinsed with 1 ml of water and this wash was also applied to the columns. The columns were then washed with 1-ml portions of a 10% solution of methanol in water and the eluents discarded. Glass tubes were placed in a rack inside the vacuum box, positioned under each extraction column. The analytes were eluted with 1 ml of methanol which was collected. The extracts were evaporated to dryness under nitrogen and the residues reconstituted in 200 μl of mobile phase for analysis. The volume injected onto the column was 150 μl.

Semi-automated procedure. The solid-phase extraction cassettes were pre-conditioned with methanol and water as in the *manual procedure*. The hexane-washed samples and standards were transferred to the cassettes, along with 0.5 ml of water used to rinse out the sample tubes. The cassettes were washed with an additional 0.5 ml of water and then positioned in the loading tray of the AASP for analysis. The purge reservoir was filled with a solution of 10% methanol in water. The AASP was programmed in the AUTO mode with respect to the first cartridge to be eluted, total number of samples, run time (25 min), cycle time (26 min), value reset (0.8 min), and number of purge pulses (10). The concentration of the compounds in each sample was determined from their peak-height ratios relative to the internal standard and the corresponding least-square line of the calibration standards.

RESULTS AND DISCUSSION

The assay conditions reported in this method evolved from the evaluation and optimization of the sample preparation, chromatography, and detection of the drug, metabolite, and internal standard from human plasma. This process began with the identification of the most selective solid-phase extraction column, i.e., the phase which was the least retentive overall but still yielded acceptable extraction efficiencies for the analytes. An important consideration in the choice of extraction columns for evaluation was the observation that indolidan contains no ionizable functional groups. Therefore, those phases which utilize ion-exchange as their primary mechanism of retention were excluded. Those tested were octadecyl (C_{18}), octyl (C_8), ethyl (C_2), phenyl, diol, cyanopropyl, and cyclohexyl. Use of the C_2 phase resulted in the cleanest extract combined with nearly quantitative recoveries. Additional selectivity was achieved through the use of a methanol–water wash prior to the elution step. A solution of 10% methanol in water was found to elute some of the plasma components without affecting the retention of the compounds of interest. The use of a 20% methanol solution resulted in the partial loss of all three analytes.

The hexane wash was added to the method to remove lipophilic substances from the plasma which began to elute from the liquid chromatographic system after 10–12 h of injections and thus interfered with subsequent chromatograms.

This step was taken only after it had been determined that the late-eluting components could not be trapped on the solid-phase cartridge by use of the valve reset feature on the AASP. This feature allows for the removal of the cartridge from the flow path to the analytical column at a preset time. At 0.8 min, which was determined to be the shortest valve time that still resulted in the quantitative transfer of the analytes, the lipophilic substances were retained. It is possible that a different phase than that employed (C_2) would provide this separation, but, as mentioned above, of the many different types of extraction columns investigated, only the C_2 resulted in the resolution required to detect the very low (< 1 ng/ml) concentrations of the drug and metabolite. The hexane wash provided a simple alternative and did not add appreciably to the time of sample preparation. To prevent the formation of emulsions and subsequent reduction in recovery, the samples were chilled briefly and then gently rotated with the hexane. Interestingly enough, cooling the samples proved to be more effective in preventing emulsions than when chilled hexane was used.

Additional experiments were performed to evaluate whether the selectivity could be enhanced by passing the sample through other solid-phase columns such as cation and anion exchange prior to the retention of the analytes on the C_2 phase. The goal was to retain other substances from plasma by a mechanism different from that used to extract the analytes. To this end, human plasma spiked with the compounds was passed through carboxylic acid (CBA), benzenesulfonic acid (SCX), aminopropyl (NH_2), and quaternary amine (SAX) extraction columns prior to the C_2 phase. The samples were acidified with hydrochloric acid prior to the cation exchange and alkalinized using sodium hydroxide before putting over the anion-exchange columns. No appreciable difference was observed in the content of the final extracts using this approach. Similar experiments were also carried out using alumina and silica solid-phase columns to pretreat the samples and also using dilute hydrochloric acid or sodium hydroxide solutions to wash the C_2 columns prior to the elution of the compounds. Again, none of these approaches resulted in any observable differences in the content of the final extract.

The choice of liquid over gas chromatography was dictated by the thermal instability of indolidan at the temperatures needed to volatilize the underivatized compound and the absence of derivatizable functional groups. For liquid chromatography, the Zorbax phenyl column was observed to provide stronger retention of the analytes and better resolution from other sample components than did either an IBM phenyl or C_{18} column. Methanol, acetonitrile, tetrahydrofuran and combinations of these solvents were evaluated as mobile phase organic modifiers; of these only tetrahydrofuran resulted in the complete resolution of the compounds from other sample constituents. The effect of pH on the separation was observed over the range 3.5–7.0 using ammonium acetate and sodium phosphate as buffer salts. The resolution of indolidan, its dehydro metabolite, and the internal standard were largely unaffected by changes in pH. An intermediate value, 5,5, was chosen so as to avoid pH extremes and thus prolong column life.

The options available for the detection of indolidan were restricted by the required sensitivity of the assay (less than 0.5 ng/ml) and the physical characteristics of the compound. Indolidan does not fluoresce, is not electroactive at

analytically useful potentials and could not be derivatized. It does possess a moderate UV chromophore. At the λ_{max} for indolidan (310 nm), however, the signal for the dehydro metabolite was only about 30% of that observed at its λ_{max} (280 nm). Rather than compromise the sensitivity of the method for either compound, a programmable UV detector was employed.

Indolidan, its dehydro metabolite, and the internal standard were all well resolved from each other and from other plasma components by this procedure (Fig. 2). The AASP allowed for the transfer of the entire sample extract onto the chromatography column thus resulting in larger responses for a given concentration than did the conventional autosampler. In all other respects, similar chromatograms were obtained by each method.

Other drugs which were concommitantly administered with indolidan were tested for potential interference in the assay. These included other cardiovascular drugs and diuretics (Table I). Although none of these drugs themselves co-eluted with the analytes, plasma from congestive heart failure patients administered several of these medications often showed interferences. No correlation was apparent between the pattern of interference and drugs administered. Indolidan and and its dehydro metabolite were successfully determined in these samples, however, by modifying the organic content of the mobile phase in each case to achieve separation. This tedious practice was not acceptable for analyzing large numbers of samples, therefore a more specific preparation scheme for these samples is under development.

Occasionally, samples contained a large amount of particulates which would clog the frits on the extraction columns. The AASP cartridges were more subject to clogging due to their stainless-steel frits. These samples were successfully treated by either centrifugation or filtration prior to transfer to the extraction

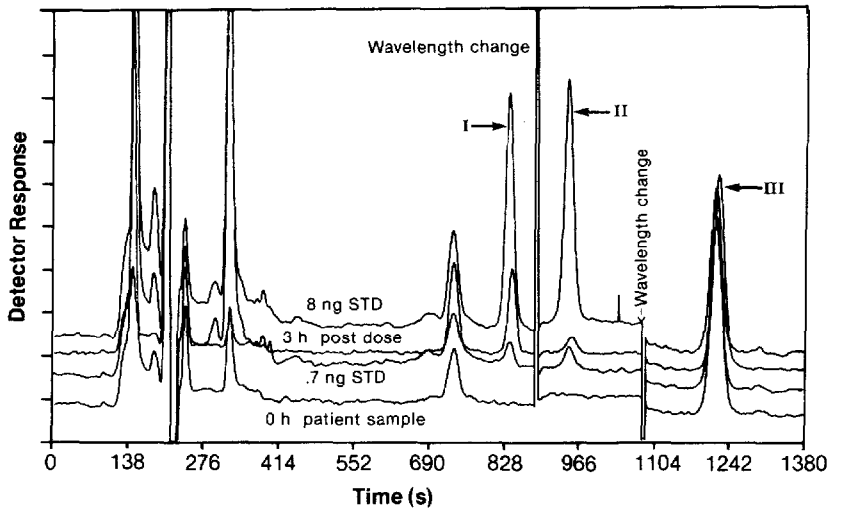

Fig. 2. Chromatograms illustrating the separation and detection of indolidan (I), its dehydro metabolite (II), and the internal standard (III) in calibration standards and in plasma from a normal volunteer adminstered a single 500-μg dose.

TABLE I

DRUGS TESTED FOR INTERFERENCE IN THE ASSAY

N-Acetylprocainamide	Acetylsalicylic acid
Allopurinol	Atenolol
Captopril	Digitoxin
Digoxin	Diltiazem
Furosemide	Hydralazine
Hydrochlorothiazide	Nifedipine
Persantine	Primadone
Procainamide	Propranolol
Quinidine	Ranitidine
Salicylic acid	Theophylline
Triamterene	Verapamil

columns. Depth filters (plastic reservoirs packed with diatomaceous earth and gauze, Analytichem International), 70-μm polyethylene frits positioned in empty plastic reservoirs, and 10-μm disposable filter tips (Zymark, Hopkinton, MA, U.S.A.) have all been successfully applied. The majority of samples analyzed in our laboratory have not required filtration.

Sample recovery

The recovery of indolidan, its metabolite, and the internal standard from plasma was determined for both methods by a comparison of peak heights from spiked plasma prepared by each procedure to solutions of the analytes in mobile phase at equivalent concentrations. For the manual procedure, the recovery was 85–90% for all three compounds. Using the AASP, the recoveries were 90–95%.

Precision and accuracy

The precision and accuracy for each procedure was determined by spiking drug and metabolite into drug-free human plasma at four different concentrations. Five replicates from each pool were assayed on each of three days. The concentrations ranged from 0.3 to 6 ng/ml for each compound. The results are shown in Tables II and III.

Similar statistics were obtained for the precision and accuracy of the assay with and without the use of the AASP. Although the accuracy appears slightly better with the AASP, it is important to note that plasma pools prepared on separate days were involved for the two procedures and this may merely reflect more accurate preparation.

Linearity and sensitivity

The relationship between the concentrations of drug and metabolite and their peak-height ratios relative to the internal standard was linear from 0.25 to at least 20 ng/ml from a 1-ml sample. The detection limit was the same for both procedures and was determined by the extent to which the detector output could be amplified and the precision of the method at that maximum amplification. A value of 0.25 ng/ml was established as the limit of detection for both indolidan

TABLE II

SUMMARY OF ASSAY PRECISION AND ACCURACY DATA FOR INDOLIDAN

In all cases, $n = 15$.

Concentration (ng/ml)	Overall R.S.D. (%)	Percentage of total variability		Overall mean	Percentage of theory
		Between-day	Within-day		
Manual procedure					
0.301	23.4	55.1	44.9	0.280	91.6
0.860	7.40	13.5	86.5	0.800	93.0
3.23	2.67	24.5	75.5	3.03	93.7
6.45	4.56	52.9	47.1	6.28	97.3
AASP					
0.310	20.4	2.4	97.6	0.290	93.6
0.826	7.18	7.0	93.0	0.780	94.4
3.10	4.19	3.6	96.4	3.09	99.7
6.19	2.83	36.1	63.9	6.13	99.0

TABLE III

SUMMARY OF ASSAY PRECISION AND ACCURACY FOR DEHYDROINDOLIDAN

In all cases, $n = 15$.

Concentration (ng/ml)	Overall R.S.D. (%)	Percentage of total variability		Overall mean	Percentage of theory
		Between-day	Within-day		
Manual procedure					
0.291	29.0	53.5	46.5	0.360	125
0.832	8.96	21.5	48.5	0.780	93.3
3.12	2.58	16.7	83.3	2.98	95.4
6.24	4.97	49.3	50.7	6.26	100.4
AASP					
0.305	34.1	59.2	40.8	0.320	103.7
0.814	6.95	3.4	96.6	0.810	100.0
3.05	3.30	13.7	86.3	3.04	99.7
6.10	3.86	17.4	82.6	6.00	98.4

and dehydroindolidan because this represented the smallest signal which could be reliably measured. Under these conditions, the signal-to-noise ratio for a chromatographic peak corresponding to 0.3 ng/ml drug or metabolite in plasma was still greater than 12 and overall relative standard deviations of 20–30% were obtained (Tables II and III).

Stability

The stability of indolidan and its dehydro metabolite in plasma was determined by spiking a known amount of each compound into drug-free human plasma and also into 0.05 *M* pH 7.4 sodium phosphate. The latter was prepared as a control,

which was stored and assayed with the plasma spikes to assure assay performance. Indolidan has been shown to be stable in aqueous solution for extended periods of time, therefore, any apparent decrease in concentration would have suggested the existence of analytical problems, not instability of the drug. Aliquots of these pools were stored in disposable glass centrifuge tubes at room temperature, +4°C, −20°C, and −70°C. Both drug and metabolite were observed to be stable at room temperature for at least five days (stability defined as ≧95% of initial remaining). The precision of the assay was decreased, however, due to the "dirty" chromatogram obtained from plasma stored at room temperature. Stability was also observed for both compounds for at least 26 days in plasma stored at +4°C, for at least two months at −20°C, and for at least five months at −70°C.

Application of the method in pharmacokinetic studies

The procedure has been used to provide pharmacokinetic data for both drug and metabolite in man following a single 500-μg dose of indolidan. Complete plasma profiles for the drug were obtained from the analysis of 0.5-ml plasma samples up to 12 h post dose, with 1.0-ml aliquots assayed thereafter.

CONCLUSIONS

Indolidan and its dehydro metabolite may be accurately determined in plasma by the described procedures. While no striking improvements in assay performance were observed by using the AASP, the convenience and time savings provided by this device were considerable. Sample preparation time was cut almost in half, from about 4 h to about 2.5 h for a batch of forty samples.

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