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Determination of Oltipraz in serum by high-performance liquid chromatography with optical absorbance and mass spectrometric detection

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ABSTRACT

Three methods have been developed for the analysis of Oltipraz in serum. A method suitable for routine use employs spiking with a homologous internal standard, off-line solid-phase extraction, high-performance liquid chromatographic separation, and optical absorbance detection at 450 nm. Method detection limit is about 1 ng/ml. A second method, less susceptible to bias from co-eluting interferences, uses a stable isotope-labeled internal standard, similar extraction and separation, and detection by thermospray mass spectrometry. Method detection limit is about 0.2 ng/ml. A third method was developed which can be used without specially synthesized internal standards. It uses on-line solid-phase extraction, with quantification by comparison with external standards. Method detection limit is about 3 ng/ml. Good agreement was observed between these methods and with similar and different methods run in other laboratories. Calibration curves were linear over the entire range which was investigated, *i.e.*, up to 500 ng/ml. Coefficients of variation were similar for all three methods, being about 5%.

INTRODUCTION

Oltipraz, 4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione, is a synthetic dithiolethione that has been found to have chemoprevention activity in breast, skin, lung, and bladder cancer animal models. Epidemiological studies have also reported that consumption of certain vegetables that are known to contain dithiolethones, namely cauliflower, brussels sprouts, and cabbage, have been associated with decreased cancer risk in humans [1]. These chemopreventive efficacy studies, together with relatively low toxicity of Oltipraz as observed thus far in animals, makes it an important compound for continued investigation as a cancer preventive agent.

This study reports on methods for measuring Oltipraz in human serum, a necessary initial step for qualifying a compound for entrance into human clinical trials. The National Institute of Standards and Technology (NIST) is responsible for providing assistance to grantee laboratories of the Division of Cancer Prevention and Con-

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trol, National Cancer Institute, in the areas of analytical method development and quality assurance. The quality assurance approach consists of circulating performance–evaluation standards to the laboratories to identify problem areas, and when problems are discovered, to provide counseling in method refinement and trouble-shooting. The development of several analytical methods for assaying serum levels of Oltipraz was necessary in the course of preparing for this program. One or more routine methods suitable for use in the clinical laboratories were needed, as well as one or more reference methods.

Solid-phase extraction of Oltipraz from serum samples using silica modified with octadecylsilane (C_{18}) proved to be quite effective, although another laboratory [2] reports the successful use of liquid–liquid extraction. Two approaches were used. The first, an off-line extraction using commercial solid-phase extraction cartridges, requires the use of an internal standard for volume correction and to compensate for non-quantitative recovery. It is desirable that the internal standard used should be chemically similar to Oltipraz, so that the extraction recovery and detector response will be about the same. This extraction technique was used with both optical absorbance detection and mass spectrometric (MS) detection.

Since the internal standards used with the extraction technique above are not commercially available, an on-line solid-phase extraction was also developed. A method based on this extraction, while somewhat cumbersome to perform with non-automated equipment, offers the possibility of doing the analysis without reference to an internal standard. It also offers some additional method independence, since the extract concentration steps are eliminated. The use of independent methods is an important means for discovering the presence of (and sources of) analytical bias.

Separation of Oltipraz from other extracted serum components was readily effected by reversed-phase chromatography on silica bonded with octadecylsilane, using a methanol–water mobile phase. Oltipraz has strong optical absorbances at 232, 305, and 450 nm. These make it quite amenable to determination by high-performance liquid chromatography with absorbance detection (LC–UV) at the levels likely to be found in serum from the NCI studies, *i.e.*, 10–500 ng/ml [2].

Analytical determination by LC–MS is also possible, since Oltipraz provides a good yield of negatively charged molecular ions, M^-, in a thermospray interface with discharge-assisted ionization. We have found that a deuterated internal standard gives both the desired co-elution and similarity of ionization mechanism for isotope dilution analysis.

EXPERIMENTAL

LC–UV method with homologous internal standard

The internal standard used in this analysis is 4-ethyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione (I.S.)[a], a homolog of Oltipraz which was synthesized in this laboratory. It has the desirable attributes of being chemically similar to Oltipraz, so that its extraction recovery should be the same, and also of having similar optical absorptivity, so that it can be used at about the same concentration as the Oltipraz.

Standard solutions were prepared by dispensing appropriate aliquots of a stock solution of Oltipraz (Rhone-Poulenc, Vitry, France) in absolute ethanol (Warner-Graham, Cockeysville, MD, USA) into 50-ml volumetric flasks. These were then made up to volume with water. Concentrations ranged between 10 and 500 ng/ml. A solution of I.S. was also made up in ethanol at about 2 μg/ml. This was used to spike both the standard solutions and samples. If the same I.S. solution is used to spike both the standards and the serum samples, the accuracy of its concentration is not critical.

Oltipraz in solution is subject to photodegradation, so that flasks should be covered, wrapped with foil, or kept in the dark when not actually in

[a] The author will make small quantities of these internal standards available to qualified investigators.

use. For storage, ethanol solutions kept in a refrigerator have been found to be stable for up to two weeks. The aqueous standard solutions are subject to possible sorptive losses of Oltipraz, and therefore should be made up fresh daily.

A calibration curve was constructed by placing 1-ml aliquots of standard solutions in vials and spiking with 50 μl of I.S. solution. The aliquots were then evaporated to 100–200 μl, either by blowing down with inert gas or by the use of a centrifugal vacuum evaporator (SVC-100H, Savant Instruments, Farmingdale, NY, USA)[a]. The concentrates were injected into the chromatograph without further treatment. Subjecting them to the same extraction–concentration process that was used with the serum samples gave identical results.

For extraction of Oltipraz from serum, 1-ml aliquots of serum were placed in vials and spiked with 50 μl of I.S. solution. The serum samples were then diluted with about 3 ml of water. This dilution has been found to increase the recovery. Sep-Pak solid-phase extraction cartridges (Waters Assoc., Milford, MA, USA) packed with C_{18} modified silica were conditioned with several milliliters of methanol followed by 2 ml of water. The spiked, diluted serum was then passed through at the rate of about 2 ml/min. It is important to keep the cartridge packing wet and not allow air bubbles to enter, except as specified later. Each cartridge was then washed with 2 ml of water and 2 ml of 40% (v/v) methanol in water. These washings remove many of the unwanted serum components. The last washing was expelled with 3 ml of air. The Oltipraz was then eluted with 2 ml of methanol followed by 3 ml of air. This eluate was concentrated by the same process used with the standard solutions. The eluate contained some water and, after evaporative concentration, the water concentration was high enough that large volumes could be injected without degradation of chromatographic peak shapes.

For the LC separations, a mobile phase of 2:1 (v/v) methanol–water containing 0.05 mol/l ammonium acetate was used. Solvent delivery was by an ISCO M2350/2360 pumping system (ISCO, Lincoln, NE, USA). Flow-rates of 1.5–2.0 ml/min were satisfactory. The buffer served to sharpen up some of the peaks from serum components which might otherwise interfere with the Oltipraz or internal standard peaks. The injector was fitted with a 1-ml loop, arranged so that liquid injected into the loop is back-flushed into the column. A pre-column of 50 μl capacity (Upchurch Scientific, Oak Harbor, WA, USA), filled with C_{18} Corasil (Waters Assoc.) packing supported on 0.5-μm frits, was used to protect the 25 cm × 4.6 mm Vydac 5-μm C_{18} column (The Separations Group, Hesperia, CA, USA). This pre-column served not only to remove components which might irreversibly sorb to the analytical column, but also acts as a filter bed. The concentrated extracts often contained visible particulate matter, but off-line pre-filtration caused serious losses of Oltipraz.

Detection was by optical absorbance at 450 nm using an ISCO V4 variable-wavelength UV–VIS detector (ISCO), with sensitivity set at 0.005 or 0.010 absorbance units full scale. If an absorption maximum at 232 or 305 nm is used for detection, the sensitivity is 33 or 70% greater, respectively, but there is a greater risk of interference from the absorbances of co-eluting serum components.

Peak heights were measured for quantification,

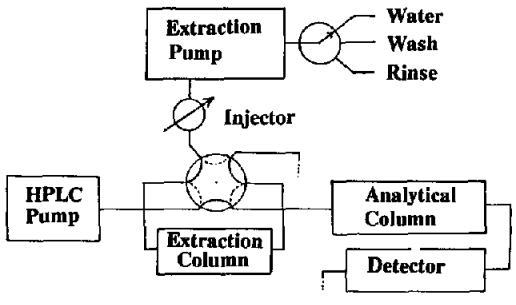

Fig. 1. Schematic of the on-line extraction system.

[a] Certain commercial products are identified in order to adequately specify the experimental procedure. This does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material or equipment identified as necessarily the best available for the purpose.

though measurements of peak areas would also serve. A calibration curve was constructed by plotting the ratio, R (where R = Oltipraz response/I.S. response), *versus* the Oltipraz concentration in the standard solution. The plot was found to be linear over the range of interest, *i.e.*, 10–500 ng/ml. Oltipraz concentrations in serum samples could then be inferred by comparing the value of the ratio, R, for each sample to the calibration curve.

LC–UV method with external standard

An additional HPLC pump (M6000, Waters Assoc.) was used to pump the samples (or standards) and washing solutions through an extraction column which was substituted for the injection loop of the HPLC system, as shown in Fig. 1. A 40 mm × 4.6 mm I.D. extraction column was constructed and packed with 10-μm μBondapak C_{18} (Waters Assoc.). This packing is somewhat less retentive than the Vydac C_{18} analytical column, so that some focusing of the extracted Oltipraz will occur when it is displaced onto the analytical column.

Serum samples and standard solutions were diluted 1:4 with water prior to injection. With water flowing through the extraction column at 2 ml/min, 1.00 ml of diluted serum or standard solution was injected. After the passage of 5 ml of water, 5 ml of washing solution containing 35% methanol in water was pumped through. The extraction column was then switched in line with the analytical column, whereupon the extracted materials were eluted. The extraction column was conditioned for the next extraction by pumping through 10 ml of absolute ethanol, followed by 10 ml of water. For quantification, peak heights from the serum samples were compared to the peak heights resulting from injections of standard solutions.

LC–MS method

A Vestec Model 201 thermospray LC–MS system (Vestec, Houston TX, USA) was used as the detector for isotope dilution determinations. The I.S. used was 4-(methyl-d3)-5-(2-pyrazinyl)-1,2-dithiole-3-thione (Oltipraz-d3), which was synthesized in this laboratory. Standard solutions, spiking, extraction, and concentration steps were similar to those used for the LC–UV method. The mobile phase was the same except that the ammonium acetate was omitted because it yielded a background ion at m/z 229, interfering with the ions from the internal standard. The flow-rate used was 1.00 ml/min. Obviously, the on-line extraction described above can also be used in this method, with the addition of Oltipraz-d3 as I.S.

The source block was operated at 275°C, and the vaporizer was operated near the "take-off" temperature, giving a tip temperature of about 210°C, and a vapor temperature of about 240°C. Discharge-assisted ionization was used, and the M^- ions of Oltipraz and Oltipraz-d3 were monitored at m/z 226 and 229, respectively. A calibration curve was constructed by the same method employed for LC–UV quantification. As a check on the integrating algorithm, calculations have been made using both peak areas and peak heights, with no significant difference appearing in the results.

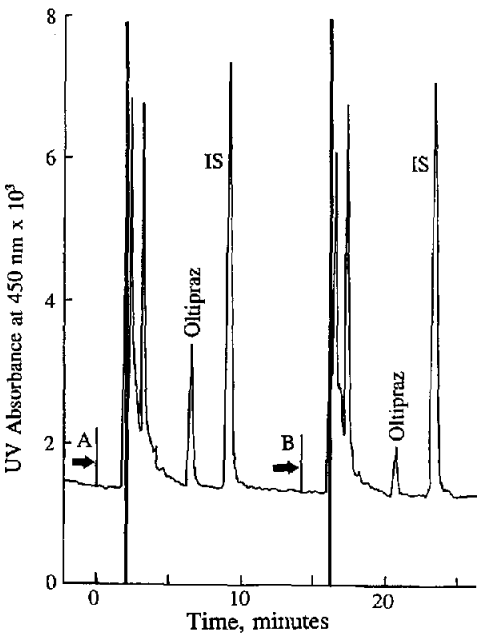

Fig. 2. Chromatograms of serum extracts. (A) Injection of the extract from a sample containing 47.7 ng/ml Oltipraz, spiked with 150 ng/ml I.S. (B) Injection of the extract from a sample containing 20.2 ng/ml Oltipraz, spiked with 150 ng/ml I.S.

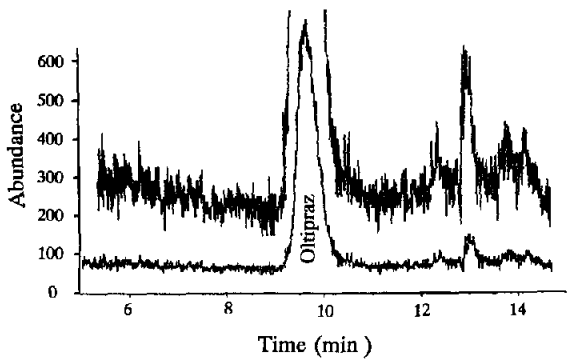

Fig. 3. Single-ion monitoring of the M$^+$ ion of Oltipraz (m/z 226) from the injection of a serum extract containing about 1 ng of Oltipraz. The upper trace is recorded at 5 × higher sensitivity.

RESULTS AND DISCUSSION

Recoveries observed in the methods using off-line extraction ranged between 50 and 90%. The losses appear to occur during the concentration of the extracts, rather than being due to inefficiency of extraction. Whether the losses are caused by oxidation, photolysis, sorption to the glass container, or some other process is not known. However, the good agreement between the three methods, together with the observation that the results show no dependence on the level of recovery, leads us to assert that the losses affect the analyte and I.S. equally and therefore do not bias the results.

A typical chromatogram obtained by the LC–UV method with I.S. is shown in Fig. 2. Other types of C_{18} columns have been tried and found to be usable when the methanol concentration in the mobile phase was adjusted to give similar retention times. Chromatograms obtained by the LC–MS method are very clean, typically showing only peaks from the analyte and I.S.

The limits of detection for each method were calculated from observations of the range of baseline noise, compared with the sensitivity. This has been found empirically to be equivalent to several other criteria [3]. Method detection limit for the LC–UV method was observed to be approximately 0.5–1.0 ng/ml, and for the LC–MS method 0.1–0.2 ng/ml. The latter can be inferred from Fig. 3, which shows an LC–MS peak from the injection of a serum extract containing about 1 ng of Oltipraz. The detection limit for the on-line extraction method is, on one hand, degraded by the smaller sample size, and, on the other hand, enhanced by the elimination of analyte losses during evaporative concentration. The resulting method detection limit is about 3.0 ng/ml.

Comparative data from all three methods are shown in Table I. Two samples from each lot of spiked serum were run in duplicate by each method. The coefficients of variation (C.V.) are typical of those observed in our analyses of many other serum samples. The agreement between the three methods is also good.

The methods utilizing internal standards have been in use in our laboratory for two years to assign Oltipraz concentrations to serum samples

TABLE I

COMPARISON OF OLTIPRAZ CONCENTRATIONS DETERMINED BY THREE METHODS

Serum No.	LC–MS, I.S.		LC–UV, I.S.		LC–UV, external standard	
	Concentration (ng/ml)	C.V. (%)	Concentration (ng/ml)	C.V. (%)	Concentration (ng/ml)	C.V. (%)
151	378	4.8	423	3.7	395	6.2
152	210	6.2	203	3.8	203	4.8
153	58.4	2.3	66.4	1.4	64.0	5.7
154	91.8	7.1	99.5	2.2	95.7	3.8

circulated in performance evaluation exercises. The median of the absolute values of relative deviations in the results reported by other laboratories using similar and different methods is about 20%.

The on-line extraction method was developed to provide a means of Oltipraz determination where specially synthesized internal standards are not available. Its lesser sensitivity could presumably be enhanced by extracting a larger volume of sample. It will not prove as well suited to routine use in clinical laboratories, however, unless the number of analyses is high enough to warrant setting up a chromatographic system with automated valving, dedicated to this analysis.

ACKNOWLEDGEMENT

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